Expression of the GLUT1 glucose transporter using the baculovirus expression system

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Award date:
1994

Awarding institution:
University of Bath

EXPRESSION OF THE GLUT1 GLUCOSE TRANSPORTER USING THE BACULOVIRUS EXPRESSION SYSTEM

Submitted by Diane L. Cope

for the degree of PhD of the University of Bath

1994

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To my parents; and to Tim, who thought "Dr. Di" sounded nice.

ACKNOWLEDGEMENTS

I would like to thank firstly my supervisors Dr. Adrian Wolstenholme, and Dr. Geoffrey D. Holman. To Dr. Holman for his invaluable injections of input into the project at all the right moments; and to Dr. Wolstenholme for his "on tap" help and guidance, without which I would never have managed.

My thanks also go to Dr. Ian Patterson and Dr. Ian Goodyer for their practical help - Dr. Goodyer for his diagrams. I would also like to thank Dr. Paul Towner for his helpful discussions and many practical tips.

My time spent at Bath has been made most enjoyable, and I have been helped by very many people from the "Middle lab", "Transport House" and others, past and present, who are far far too numerous to mention by name.

Finally, and most essentially, I would like to thank Adam. His patience and love through the tough bits, and industrious help through the determined bits have been invaluable in both the bench work and writing involved in the course of this project.

CONTENTS

ABSTRACT

The Baculovirus Expression System has been used to express the mammalian glucose transporter isoform GLUT 1. The full-length protein was expressed to a level of ~ 200 pmol/mg membrane protein, and was found to exhibit a lower apparent molecular weight (~50 kDa) than the native transporter derived from human erythrocyte membranes. Photolabelling studies using transporter side-specific ligands demonstrated that the expressed protein binds the bismannose compound 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxyl)-2-propylamine (ATB-BMPA) and cytochalasin B at its exofacial and endofacial binding sites respectively.

Constructs of GLUT 1 which produce either the N-terminal (amino acids 1 - 272) or C-terminal (amino acids 254 - 492) halves of the transporter are expressed at levels in the plasma membrane which are similar to that of the full-length GLUT 1, but do not bind either ATB-BMPA or cytochalasin B. A dual infection approach has been used to demonstrate that when Sf9 cells are simultaneously infected with recombinant baculoviruses encoding both the C- and the N-terminal halves of GLUT 1, then the ligand labelling is restored. Immunoprecipitation using specific antibodies demonstrates that the labelling is associated only with the C-terminal half. This suggests that labelling of this domain is dependent on the presence of the N-terminal half of the molecule. This supports the bilobular model of the three-dimensional structure of the GLUT 1 protein, in which separate C- and N-domain halves pack together to produce a ligand-binding conformation.

ABBREVIATIONS

AcMNPV	*Autographa californica* multiple nuclear polyhedrosis virus
ASA-BMPA	2-N-(4-azidosalicoyl)-1,3-bis-(D-mannos-4-yloxy)-2-propylamine
ATB-BMPA	2-N-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine
ATP	Adenosine triphosphate
ave.	average
BCA	Bicinchonic acid
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary cells
DMF	Dimethylformamide
ddH_2O	Double distilled water
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
GLUT	Glucose transporter isoform
HepG2	Hepatoma G2
IAPS	Iodo-4-azidophenylamido-7-O-succinyldeacetyl
K_d	Dissociation constant
M_r	Molecular mass
moi	Multiplicity of infection
mRNA	Messenger ribonucleic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

pfu	plaque forming units
Sf9	*Spodoptera frugiperda Clone 9*
SDS	Sodium dodecylsulphate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylene diamine
TES	Tris/EDTA/sucrose bufffer
Thesit	Nonaocta (ethylene glycol) dodecyl ether, $C_{12}E_9$
TM	Transmembrane segment
Tris	Tris-(hydroxymethyl)-methylamine
Tween-20	Polyoxylethylenesorbitan monolaurate
UV	Ultraviolet
V_{max}	Maximal velocity.
X-gal	5-bromo-4-chloroindol-3-yl β-D-galactoside

CHAPTER 1

INTRODUCTION

1.1 FACILITATED GLUCOSE TRANSPORT

Glucose is a major source of metabolic energy, and its movement across the plasma membrane of cells is central to the requirements of many organisms. Virtually all mammalian cells have a system of facilitated transport of glucose, allowing the movement of glucose down its chemical gradient, either into or out of cells. The transport is stereospecific, and in mammals is mediated by a family of distinct but related transmembrane transport proteins (Bell et al, 1990).

That a family of transporters exists, rather than a single universal isotype, reflects the variations in glucose requirements, and the homeostatic roles of different cell types.

To date, at least six genotypically distinct transport isotypes, each characterised by unique tissue distribution, regulation, and kinetic properties have been cloned and identified in mammalian tissue. The family members are closely related in terms of primary, and proposed secondary structure within the membrane. Such similarities are not limited to mammalian transporters, with significant amino acid sequence homology and proposed two- dimensional structural similarities also found in the sugar transport systems of protozoa, algae, yeast and prokaryotes (Baldwin, 1993 provides a comprehensive reveiw of sequence homologies between prokaryotic and eukaryotic hexose transporters)

The particular mechanism used by cells for the uptake or release of glucose is reflected by specific needs, related to the environmental glucose concentrations. That most mammalian cells utilise the facilitated diffusion systems which will be described in this thesis, is reflective of fairly reliable glucose concentrations. For example, red blood cells (RBC) are normally permanently bathed in ~5mM glucose. Hence there is little requirement for transport of glucose against its concentration gradient.

1.1.2 THE MAMMALIAN GLUCOSE TRANSPORTER FAMILY

1.1.2.1 GLUT 1

The most extensively studied mammalian glucose transporter is that present in human erythrocytes. To date, it is the only one to have been purified (Baldwin et al 1982; Baldwin and Lienhard, 1989), and this has allowed partial amino acid sequencing, and the generation of specific antibodies (eg. Davies et al, 1987), which has enabled the isolation of a cDNA clone (Mueckler, 1985; Birnbaum et al 1986). The gene encoding this protein has also been isolated (Fukumoto et al 1988; Williams and Birnbaum 1988). Although this transporter is abundant in erythrocytes, comprising up to 5% of the membrane protein; it is not exclusive to these cells; GLUT 1 mRNA is found in almost all human tissue (Flier et al, 1987b; Kahn and Flier, 1990). GLUT 1 is present in the highest levels in the brain, and abundantly found in blood barrier tissues, especially the blood-brain barrier, the placenta, the retina, and the kidneys. Significant levels of GLUT 1 protein are also present in insulin sensitive skeletal and cardiac muscles, and in fat cells. The near ubiquitous tissue distribution of GLUT 1, and presence of other isoforms of transporter in the same tissues in many cases, has led to the suggestion that GLUT 1 is the "basal" glucose transporter of many cells (Thorens et al, 1990).

Since the cloning and sequencing of the human erythrocyte glucose transporter, analogous sequences have been determined for rat, mouse, rabbit, chicken, and pig . Each of them are highly homologous to each other, and to human GLUT 1, at the amino acid level. For example, the rat sequence has 98% homology with the human, and the rabbit sequence 97%. Such a high degree of homology suggests that all parts of the molecule are functionally important.

GLUT 1 mRNA and protein are also found in highly elevated levels in transformed mammalian cell lines in culture. Although this observation has been made for very many cell lines (Warburg, 1923; Flier et al, 1987; Hiraki et al, 1989; Birnbaum et al, 1987; Merrall et al, 1993), particularly high levels are found in the Hep G2 cell line (Mueckler et al, 1985). Consequently GLUT 1 is often referred to as the Hep G2-type glucose transporter, named according to this source. The observation that GLUT 1 expression levels are elevated when cells are transformed to culture lines has led to the suggestion that many, if not all, circulating growth factors (as are abundantly present in cell culture media), as well as the depravation of glucose, can stimulate GLUT 1 expression (Weber et al, 1984; Rollins et al, 1988; Hiraki et al, 1988; Kahn and Flier, 1990; Haspel et al, 1986; Ortiz et al, 1992). It has been shown that an increase in glucose uptake accompanies normal cellular proliferation (Weber et al, 1984). Under normal cicumstances, glucose uptake will return to the resting level following this. A number of tumour states also demonstrate elevated GLUT 1 expression (Yamamoto et al, 1990).

The kinetics of GLUT 1 have been found to be asymmetric (Walmsley 1988; Lowe and Walmsley, 1986.). The asymmetry has been suggested to be due to a high proportion of carriers having an inward-facing conformation, as occurs at low temperatures (Lowe and Walmsley, 1986). However, the ratio of inward- facing to outward- facing transporters has been found to be roughly the same at physiological temperatures. Although there is only a 2.5 fold difference in affinities for glucose between the inward and outward- facing conformations, the asymmetry is conferred by the net efflux K_m of glucose being about ten times higher than the net influx. That both influx and efflux K_m's are significantly lower than the equilibrium exchange K_m is very important in transporter function, and is discussed in section 1.1.4.

These observations, together with the known tissue distribution of GLUT 1, have led to the proposal that the salient property of this isotype is that

it can function efficiently as a unidirectional transporter under low external glucose conditions, where intracellular glucose demand is high (Gould and Holman, 1993).

1.1.2.2 GLUT 2

On the basis of very low levels of GLUT 1 mRNA detectable in the liver, and the observation of very different glucose transport kinetics in hepatocytes, it was proposed that a genotypically distinct glucose transporter was functioning in the liver (Elliot and Craik, 1982; and Axelrod and Pilch, 1983). On the basis of this, the search for another glucose transporter was made specifically in liver cells. "GLUT 2" was cloned by Fukumoto and co-workers, and by Thorens and co-workers, in 1988; by using the GLUT 1 cDNA to probe gene libraries from hepatocytes. The new transporter was found to have 55% sequence homology with GLUT 1; but hydropathy plots, used to predict secondary structure, show the two to be virtually identical, suggesting a close similarity in general molecular structure.

As well as being expressed at high levels in the liver, it is also found in very high levels in the β-cells of the pancreas, where it is thought to be involved in the glucose stimulated release of insulin (Orci et al, 1989; Thorens et al. 1990.). GLUT 2 is also expressed in the basal (blood system) side of the small intestinal epithelium; acting in conjunction with a glucose/Na^+ symporter which actively transports glucose into the cells from the apical (digestive tract) side; the GLUT 2 acting to rapidly export glucose from its side of the cells to the blood. The expression of this transporter in kidney epithelia is thought to perform a similar function. This isoform therefore plays an important overall part in blood glucose homeostasis. The markedly different role of GLUT 2 to that of GLUT 1 is reflected in its different kinetic profile. GLUT 2 shows symmetrical transport (Craik and Elliot, 1979), and has a high K_m for the glucose analogue 3-O-methyl-glucose (~42mM) (Gould et al, 1991); which

means that, under physiological glucose concentrations, glucose flux is proportional to glucose concentration, and there is therefore no transporter saturation, which would be rate limiting. A high transport capacity, and no effective rate limitation provide appropriate characteristics for a transporter expressed in the situations occupied by GLUT 2.

1.1.2.3 GLUT 3

The GLUT 3 glucose transporter was isolated using a similar approach to that used in the discovery of GLUT 2; by the screening of a foetal muscle library (Kayano et al, 1988). Its expression is at high levels only in the brain, nerves and heart; and it may act in conjunction with GLUT 1 to meet the high glucose demands of these tissues (Gould et al, 1991; and Shepherd et al, 1992). GLUT 3 is also found in muscle, fat, liver, kidney and placenta, at very low levels. Since its general role seems to be primarily as glucose transporter to high- glucose demanding tissues, its presence in these tissues is thought to be limited to specific localised areas of cell types having such glucose demand (Gould and Holman, 1993).

The kinetics of this transporter are such that tissues expressing it will, in conditions of low glucose concentration, receive glucose in preference to tissues expressing other isoforms. GLUT 3 shows a low Km for exchange flux for 3-O-methyl-glucose (~10mM) (Gould et al, 1991). Since GLUT 1 is the primary isoform present in the blood/brain and blood/nerve barriers, and it exhibits a higher Km for equilibrium exchange than GLUT 3; under normal conditions it seems that the presence of GLUT 3 acts as a backup system. However, in times of hypoglycaemia, or increased glucose demand, the low Km of GLUT 3 for hexoses allows the successful uptake of glucose from low blood glucose concentrations (Gould et al, 1991). GLUT 3 has also been cloned from mouse and chicken. The extent of the cross-species sequence homology is high.

1.1.2.4 GLUT 4

GLUT 4 (originally cloned by five independent laboratories simultaneously, James et al, 1989; Birnbaum, 1989; Charron et al, 1989; Kaestner et al, 1989 and Fukumoto et al, 1989) is expressed exclusively in insulin-responsive tissues (adipocytes, skeletal and cardiac muscle). Stimulation of these cells with insulin can produce up to a 30-fold increase in the rate of glucose transport in some tissues (Ploug et al, 1987). The control of this transporter is also by circulating glucose levels. In insulin-deficient, glucose starved states, adipocyte levels of immunologically detectable GLUT 4 at the cell surface, are found to become reduced (Berger et al, 1989).

The insulin-induced increase in glucose transport by GLUT 4 is brought about in two principal ways. Firstly, the action of insulin is to apparently increase the V_{max} of the transporter, whilst barely altering the K_m value (Dohm et al, 1988; Palfreyman et al, 1992; Keller et al, 1989), though how a proportion of this is accomplished is, as yet, unclear. Unlike GLUT 1, the kinetic properties of GLUT 4 are symmetrical, with Km values of 2 to 5 mM (Thorens et al 1988; Fukumoto et al, 1988). The most salient property of this transporter, however, is its localisation in intracellular vesicles under normal, non-insulin stimulated conditions. The second action of insulin then, is to bring about the translocation of these transporters to the cell surface, which leads to much of the observed increase in V_{max}. The low K_m of this transporter would then ensure a maximal operation rate, for the rapid removal of glucose from the blood into the cells. In an insulin stimulated state, glucose transport is not rate-limiting to metabolism (Dohm et al, 1988). GLUT 4 sequences have also been determined in rat and mouse, to reveal a high degree of homology to one another and to human (rat to human GLUT 4 homology 95%).

1.1.2.5 GLUT 5

The GLUT 5 protein has recently been isolated (Kayano et al, 1990), and had been found to be a high affinity fructose transporter, expressed to high levels in the small intestine, testes, spermatozoa, muscle, brain, and adipose tissue. Where GLUT 2 is localised on the basolateral border of the cells of the small intestine epithelium, and the unrelated glucose/Na^+ symporter is localised at the apical border of the same cells; GLUT 5 also appears to be specifically located on the apical (digestive tract side) border (Davidson et al, 1992). Where the glucose transport functions are carried out by the symporter and GLUT 2; GLUT 5, having a high affinity for fructose, and only a limited affinity for glucose, is considered to be responsible for the uptake of dietary fructose (Burant et al, 1992). GLUT 5's role as a fructose transporter and not a glucose transporter would explain its distribution in tissues well supplied with the other transport isoforms. Despite this obvious specificity difference, GLUT 5 shares significant sequence homology with the other family members.

1.1.2.6 GLUT 6

GLUT 6, a glucose transporter-like sequence, named according to chronological convention, was identified, and mRNA detected in all tissues investigated (Kayano et al, 1990). However, analysis of the sequence found that, despite having a high degree of homology with GLUT 3 (79.6%); the cDNA reveals multiple stop codons and frame shifts. The high degree of identity with GLUT 3 suggests that the glucose transporter-like region of the GLUT 6 pseudogene arose as a result of a viral reverse transcription event, causing the insertion of a GLUT 3 mRNA transcript into the non-coding region of a ubiquitiously expressed gene (Kayano et al, 1990). Time, and no requirement for a protein product, have allowed extensive mutation; and it is very unlikely to produce a functional transport protein.

1.1.2.7 GLUT 7

This most recently isolated member of the transporter family shares 68% sequence identity with GLUT 2. It is believed that GLUT 7 is responsible for the intracellular transport of glucose in liver cells, across the endoplasmic reticulum (ER.) membrane, into the cytosol (Waddel et al, 1992). In the liver, glucose is produced by gluconeogenesis and the degradation of glycogen, for export into the blood. The final step in both processes is the removal of a phosphate from glucose-6-phosphate, by glucose-6-phosphatase. This multicomponent enzyme resides in the ER, and the synthesised glucose is initially confined to the lumen of the ER; hence the role as the intracellular glucose transporter.

The very close sequence similarities of GLUT 7 to GLUT 2 (100% in places) may be significant in the origin of GLUT 7. An absence of base drift in the third position of many of the codons suggests that GLUT 7 is either a complex splice variant of GLUT 2 (a simple variant would have the 100% identity regions corresponding to intron-exon junctions, which is not the case), or has an evolutionary origin in GLUT 2. There is one area of significant difference between GLUT 7 and GLUT 2: a unique six amino acid sequence at the C-terminus of GLUT 7. This sequence motif (KKMKND) is a consensus sequence for the retention of membrane-spanning proteins in the ER.

1.1.2.8 NON-MAMMALIAN SUGAR TRANSPORTERS

Many non-mammalian glucose-transporter- type proteins have been identified. From sequence homologies, it is clear that the general 12-membrane-spanning-domain structure is found, in some form, in representatives of most types of organism. However, there can be some important functional differences between these transporters and the general GLUT 1- based model. Substrate specificity is varied. Although many have a

high affinity for D-glucose, some are more specifically fructose transporters (such as GLUT 5), or lactose (e.g. the LAC P transporter of *Kluyveromyces Lactis,* Chang and Dickson, 1988), D-galactose (eg. the GAL 2 of *Saccharomyces cerevisiae*, which also has a high affinity for glucose - Szkutnicka et al, 1989),or maltose (eg. MAL 6T of *Saccharomyces cerivisiae,* Hong and Marmur, 1986). Some transport pentoses, such as L-arabinose (e.g. the Ara E of *Escherichia coli,* Maiden et al, 1988). Some members of this transporter family are transporters of a non-sugar substrate; the six-membered ring compound quinate (such as the QUTD gene for quinate permease, allowing *Aspergillus nidulans* to exist on quinate as a sole carbon source, Hawkins et al, 1988).

Although homologous to the mammalian model transporter in sequence, structure, and substrate specificity, a number of transporters, particularly in prokaryotes, have a different mechanism of function. For example, the Gal P gene of *E. coli* encodes a transporter which does not function by passive diffusion, but by active transport of glucose against its concentration gradient. This is achieved by the simultaneous transport of a single proton, per glucose molecule. This is therefore a glucose/H^+ symporter. This is reflective of the difference in environmental glucose availability of an organism such as *E. coli*, compared to mammalian cells. Where a mammalian cell will usually be bathed in a good concentration of glucose (~5mM in blood), glucose concentration can be very variable for a bacterium, possibly very low - hence the the development of this particular adaptation.

1.1.3 GLUCOSE TRANSPORTER STRUCTURE

1.1.3.1 GENERAL STRUCTURE

The family of mammalian glucose transporters, and many other hexose transporters, share a common basic molecular structure. Most, like GLUT 1 (from which the basic structure was derived, from HepG2 cell glut1, Mueckler et al, 1985) are about 500 amino acids long , with mobilities of between 45 and 55 kDa on SDS-PAGE.

The principal structural similarity between the members of the glucose transporter superfamily is that there are twelve predicted membrane spanning regions of a-helix, with both termini in the cytoplasm (this has been confirmed by the use of antibodies made to peptides directed towards areas of the N and C termini, and the finding that they cannot recognise these areas when only the external surface of the cell is available for binding, (Davies et al, 1987, 1989). There is a large loop between the first and second transmembrane domains (TM1 and TM2), and a large cytoplasmic loop between helixes 6 and 7. The remaining helix connecting loops are relatively short. This basic structure is depicted in figure 1.1. It is based on GLUT 1, and shows regions and residues that are highly conserved throughout the transporter superfamily.

Structurally the most significant area of difference between members of the family is that of overall length. The primary areas of variation are the amino terminal tail (varying from 10 residues in mammalian GLUT 2, to more than 90 residues in a yeast transporter), and the extracellular loop region between helices 1 and 2 (from 19 to 66 amino acids, 33 in GLUT 1). Apart from these differences, the overall structure varies only moderately between different transporters, with some areas of very high conservation, particularly between the mammalian isoforms.

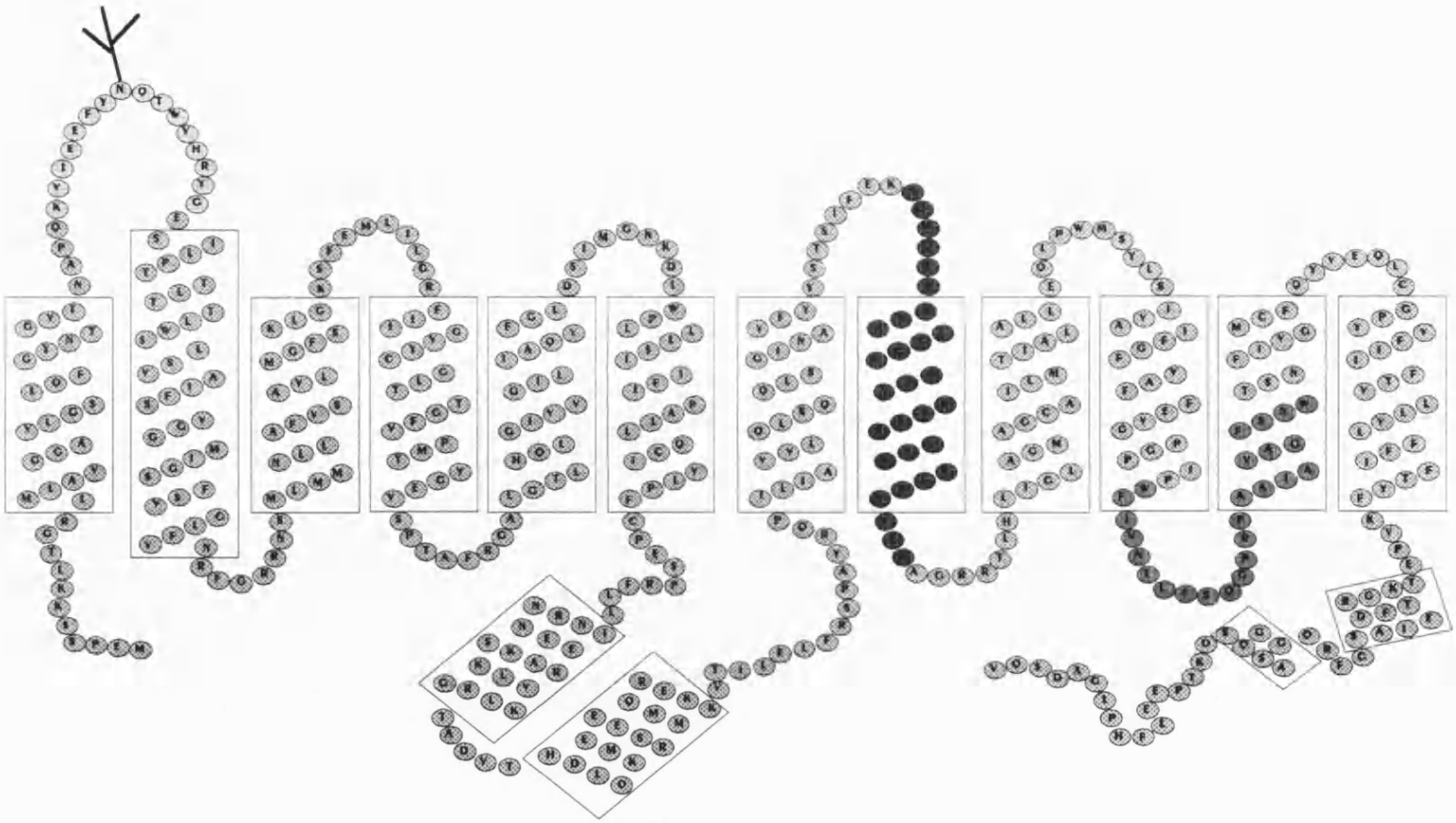


Figure 1.1 Schematic representation of the proposed two-dimensional structure of human GLUT1. Proposed sites of ligand binding are shown.

The large central loop, and the very short nature of the remaining a-helix connecting loops may be indicative of a bilobar tertiary structure. The length and sequence of many of the connecting loops, especially at the cytoplasmic surface, are highly conserved, at seven to 15 residues, and are generally shorter at the cytoplasmic surface. This suggests a very close packing of the six helixes of each domain, rather than a single bundle of twelve helixes. The slightly shorter loops on the cytoplasmic surface suggest a closer association of the helixes at this side of the molecule. A structure of this nature has been observed in the twelve -membrane- spanning domain protein of *E.coli*, lactose permease, by low resolution electron microscopy (Li and Tooth, 1987). A molecular model of the two domain packing is illustrated in figure1.2 (from Hodgson et al, 1992, with permission from Dr. G.D. Holman)

The probability of the glucose transporter structure existing as two six-helix lobes raises the suggestion that the structure may be the evolutionary result of the duplication of a gene encoding a protein which spans the membrane with six helixes. The repetition of two conserved motifs in the two halves of the molecule also supports this view. The GRR(K) motif is found between TM2 and TM3 in the N-terminal half, and between TM8 and TM9 in the C-terminal domain (Henderson, 1991) . The motif EXXXXXXR is found between helixes 4 and 5 of the N-terminal domain, and again in the loop connecting helixes 10 and 11. That these features are seen in the sequences of yeast and bacterial transporters suggests that if gene duplication is the case, it must have occurred early on in the evolution of the gene. However, these sequences may only serve to indicate the maintenance of conformational stability. Structural features such a b-turns have been suggested as an explanation for the GRR motif, since this has also been found in unrelated multi-membrane spanning proteins (Maiden et al 1987, Henderson 1991). They may be involved in salt-bridging between the helixes. Nevertheless, this need not be contrary to the suggestion of an ancestral gene duplication.

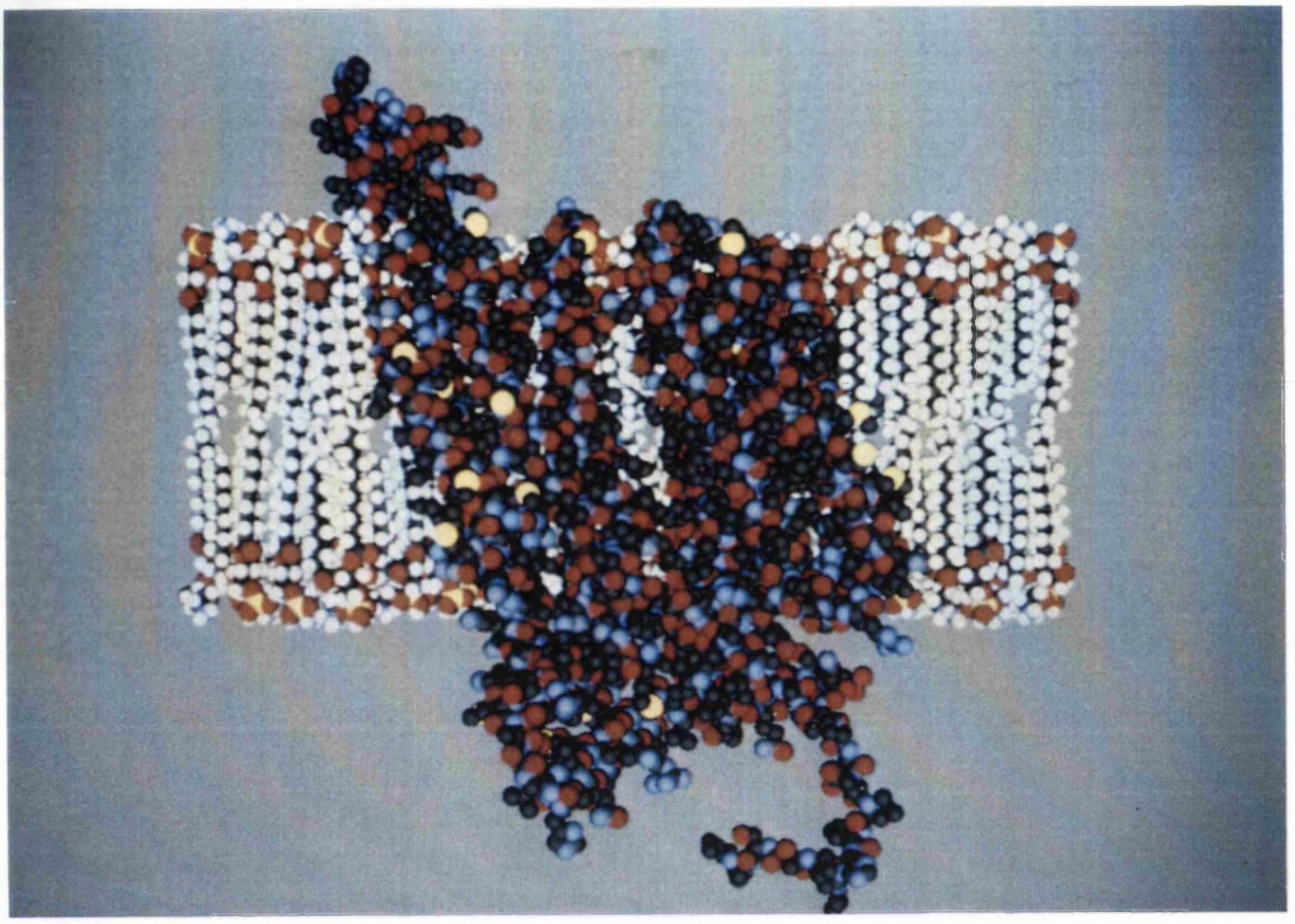


Figure 1.2 Molecular model of the three-dimensional structure of GLUT1. Reproduced with permission from G.D.Holman, based on molecular modelling work of Hodgson et al, 1992.

Analysis of GLUT 1 by infra- red spectroscopy suggests that more than 80% of the structure is α-helical (Chin et al, 1986; and Alvarez et al, 1987), and that most of this structure resides within the lipid bilayer, although the highly hydrophilic central loop region between TM6 and TM7 is predicted to contain a large portion of α-helix located cytoplasmically (Henderson 1991). Despite this, 80% of the residues have been found to be accessible to deuterium exchange, suggesting that most of the molecule is accessible to water (Gould and Bell, 1990). This is consistent with the existence of a pore formed by the transmembrane regions, through which glucose can travel. Other structural features also support the hypothesis of a glucose pore. Molecular modelling suggests that most of the highly conserved residues of the helical portions, are on the faces of the helixes predicted to be towards the centre of the protein, away from the membrane lipid. The amphipathic and highly conserved nature of TM7 has been proposed to be evidence for the formation of a transmembrane channel (Mueckler et al, 1985). Molecular modelling shows that adjacent to the highly conserved region of TM7 is a series of conserved threonine and asparagine residues in TM8, which are also considered to form part of the hydrogen bonding channel concerned with the movement of glucose.

The GLUT 1 glucose transporter is a glycoprotein, glycosylated at a single point of the molecule, which was found by the use of proteolytic cleavage and a series of antibodies, to be located on the extracellular domain on the loop between TM 1 and TM 2 (Cairns et al, 1987, Flier and Kahn, 1990). The glycosylation is heterogeneous, hence the broadness of the band on SDS-PAGE. The carbohydrate represents about 15% of the weight of the transporter (Sogin and Hinckle, 1978). The glycosylation site has been found to be Asn 45 (Mueckler et al, 1985). Endoglycosidase F treatment of detergent solubilised purified transporters results in a very sharp band of M_r 46,000 Da on electrophoresis, which represents a virtually deglycosylated form (Lienhard

et al, 1984); though treatment with endoglycosidases in concert shows that the mature protein has a core polypeptide of M_r 38,000 on electrophoresis (Haspel et al, 1985). The structure of the asparagine-linked sugar chain of GLUT 1 from human erythrocytes has been fully determined (Endo et al, 1990). Both high- mannose type and complex trimmed, and trimmed and decorated sugar chains are found in the molecule. Studies of GLUT 1 expressed in a mutant form of CHO cells, deficient in N-acetylglucosaminyltransferase 1 activity show that altered asparagine- linked glycosylation can effect the biogenesis of the transporters, but does not affect glucose uptake (Haspel et al, 1988). It has been suggested that the alterations may cause changes in targetting, or disturb the formation of a functionally appropriate conformation, rather than a more obvious effect on transport activity (Asano et al, 1993)

1.1.3.2 Two Glucose Binding Sites.

Other areas of good sequence conservation are those that have been implicated in the binding of glucose. It is generally accepted that there are two discrete glucose binding sites, an intracellular and an extracellular. Each has specific ligands, which are not recognised by the other, and these are useful tools in labelling the binding sites, and even locating transporter molecules. These are discussed in more detail in chapter 5. The specific nature of such ligands permits their use in determining ligand recognition, in conjunction with mutagenesis studies.

In the C-terminal half, a conserved sequence, PESPRFL, located between TM6 and TM7 may serve a role in substrate recognition (Henderson, 1990). Close to this, in helix 7, is a twelve residue motif that is conserved in all the mammalian isoforms, and largely conserved in other superfamily members, which is thought to be concerned with both substrate binding, and pore formation. If the first glutamine of this motif (Gln -282) is mutated to a leucine (in GLUT 1), the binding of the ligand ATB-BMPA (a bis-mannose

derivative), specific to the external glucose binding site does not occur, and the rate of glucose transport is reduced by half (Hashiramoto et al, 1992). Also close to this sequence is a three residue motif, QLS, that is conserved in GLUT 1, GLUT 3 and GLUT 4 (as well as transporters from other species), having high affinity for D-glucose, but is not found in GLUT 2, GLUT 5, or other superfamily members having higher affinities for D-fructose than for D-glucose. Since the principal difference between D-glucopyranose and D-fructofuranose is the anomeric position, at C1 for D-glucopyranose, and C2 for D-fructofuranose; this motif is probably involved in the binding of the C1 position of D-glucopyranose, conferring specificity for this.

1.1.4 GLUCOSE TRANSPORTER FUNCTION

The accepted sequence of events for the transport of glucose across a membrane by a facilitative glucose transporter is as follows. A glucose molecule, presented to the transporter in the appropriate orientation, is recognised by and bound to the glucose binding site on that side of the membrane. This binding causes a conformational change in the transporter such that the glucose is passed to the opposite side of the molecule, and released. The orientation of the glucose remains the same throughout, and must present the opposite-side binding site in the reverse orientation to bind. The nature of the conformational change during transport is such that only one binding site is available for ligand binding at a time. The conformational change involved in transport results in the opposite-side site becoming available for binding. This is known as the alternating conformer model of glucose transport, and all aspects of it have been experimentally demonstrated.

1.1.4.1 TWO SEPARATE GLUCOSE BINDING SITES

Studies using the side-specific ligands to label the transporter have been used to suggest some structural separation of the internal and external

binding sites. The extracellular-side specific bis-mannose labelling site has been mapped to TM9 (Holman and Rees, 1987), and TM8 (Davies et al, 1991). The cytoplasmic-side specific ligand, cytochalasin B, a fungal metabolite, has been shown to bind an area between TM10 and TM11 (Holman and Rees, 1987; Davies et al, 1991; and Cairns et al, 1987). Both sites, to note, are resident of the C-terminal half of the transporter.

Cytochalasin B (Taverna and Langdon, 1973; Lin and Spudich, 1974) specifically binds to the cytoplasmic glucose binding site. It inhibits glucose transport when presented at the cytoplasmic side of the membrane, in competition with glucose. It's inhibition of glucose influx is non-competitive when present at the internal side. This is evidence for the alternating conformer model, suggesting that when cytochalasin B is bound, the transporter is locked into the cytoplasmic-site orientation

ATB-BMPA irreversibly labels the protein from the external glucose binding site, inhibiting transport by locking the transporter in the extracellular-facing conformation .

Forskolin is also a potent inhibitor of glucose transport, and a tritiated form is used as a photoaffinity reagent for the glucose transporters of erythrocytes (Shanahan et al, 1987; Wadzinski et al, 1988). Binding is cytochalasin B inhibitable, by binding the cytoplasmic-side hexose binding site.

Site-directed mutagenesis has been used to determine specific features involved in transport. The truncation of 37 amino acids (of 42) of the C-terminal tail results in a transporter with a low affinity for extracellular ligands (E.g. ATB-BMPA), and no transport activity, in GLUT 1 expressed in CHO cells (Oka et al, 1990). A loss of ATB-BMPA labelling is also produced by the mutation of the highly conserved Gln-282 of the binding site (TM7), though this also causes a 50% reduction of internal-side specific ligand binding (Hashiramoto et al, 1992).

Single residue mutations near to the proposed binding sites for both the internal and external ligands have shown that loss of labelling of only one of the ligands results. Such studies have also demonstrated varying effects on transport efficiency (Garcia et al, 1992; Oka et al, 1990, amongst others).

1.1.4.2 Conformational Changes

A conserved triple proline motif (GPGPIP) in TM10 is considered to be a point of considerable flexibility, and as such is believed to be central to the conformational changes between the external and internal-facing transporter forms (Gould and Bell, 1990). Molecular modelling and dynamic studies have supported this, placing particular emphasis on the last two proline residues (Pro 383 and Pro 385)(Hodgson et al, 1992; unpublished). Cytochalasin B (a competitive intracellular glucose transport and binding inhibitor) is thought to bind to this region, which is therefore important in the binding site, and the prevention of state change on inhibitor binding (Holman and Rees, 1987). That the removal of the C-terminal domain results in an inward-facing -locked state is indicative that this domain may also be important in the switching mechanism from one form to the other (Oka et al, 1990).

Much of the kinetic data for the GLUT 1 glucose transporter is also indicative of a conformational change between two conditions. This is based on the observation that sugar exchange across the membrane is faster than either sugar uptake, or the movement of glucose out of a cell (Widdas, 1980). That is, the return of a transporter to the original conformation is much slower (and therefore rate-limiting) without substrate presence, than the substrate binding-induced change which occurs upon glucose exchange.

Further evidence for conformational change is that the inactivation of the transporter by the labelling of a modifying agent FDNB is faster in the presence of substrate (sugar). That is, FDNB (1-fluoro-2,4-dinitrobenzene) inactivates GLUT 1 sugar transport in RBC. This inactivation is faster in the

presence of D-glucose, mannose, or 2-deoxy-D-glucose (transported sugars) (Krupka, 1971). The presence of a non-transported sugar, maltose, protects the molecule from FDNB labelling. FDNB specifically binds to the cytoplasm-facing conformation of the transporter. This has been demonstrated by the fact that C1, C2 and C3 alkyl substituted glucose analogues enhance FDNB labelling; but C4 or C6 alkyl substitutes either reduce labelling, or have little effect. (Barnett et al, 1975). That is, when transportable substrate is present outside the cell, the transporter rapidly changes conformation to the intracellular-facing form, on binding and transportation from the outside. This makes sites available to FDNB labelling. When no substrate is available for influx, or only inside-specific derivatives are available, the rate of conformational change towards the inside of the cell is much slower, and so therefore is FDNB labelling.

1.1.4.3 GLUCOSE ORIENTATION

The use of glucose analogues derivatised at different positions of the molecule has shown that the movement of glucose through the transporter is orientation dependent, and provides an insight into the specificity of the two sites for different areas of the molecule, and therefore the specificity of different molecules as competitive transport inhibitors. When glucose is derivatised at the C1 position (e.g. n-propyl-β-D-glucopyranose) it is a good competitive inhibitor of glucose transport when present on the cytoplasmic side, but inhibits only poorly from the extracellular environment. The model of glucose transport shown in figure 1.3 depicts glucose binding to the internal binding site (part b). From this, it is clear that a C1-derivatised molecule could not bind well to this conformation, because of steric hindrance, but could bind the inside-facing conformation. It is also clear that steric interference would prevent such a molecule from being transported. Sugars derivatised at the C6 position (e.g. 6-O-propyl-D-galactose, 6-O-propyl-D-glucose, and 6-O-pentyl-

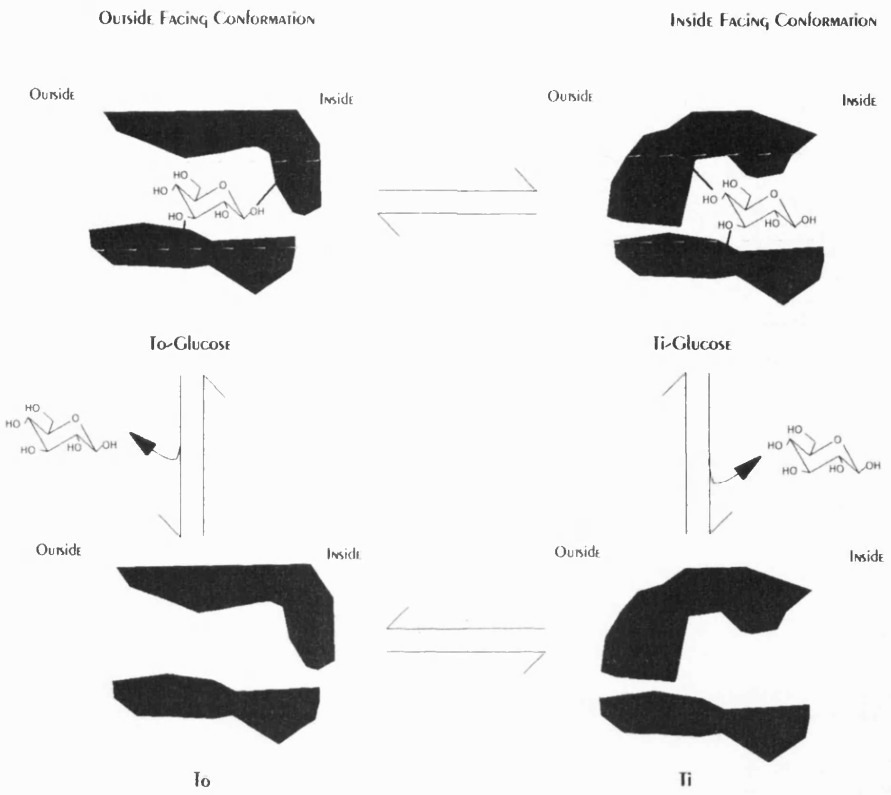


Figure 1.3 The two state carrier model for the human erythrocyte glucose transporter. Glucose can bind to either the inward or outward conformation.

D-glucose) are found to be good inhibitors of glucose influx, and bind only poorly to the internal site (Barnett et al, 1973, 1975).

The model shown in figure 1.3, and the model of Barnett et al 1975 shows the model of transport based on derivatised sugar molecule studies. This shows that the glucose molecule approaches the extracellular binding site with the C1 end towards the binding site. The binding, by hydrogen bonding and dissociation of water from the bonding residues causes a conformational change, such that the glucose moves through an essentially hydrophobic channel, and the transporter molecule closes around the C4/C6 end of the glucose, and opens at the C1 end, such that the cytoplasmic binding site is now open, and the periplasmic site is closed (Barnett *et al*, 1975). The glucose molecule is released, and the internal site receptive to a glucose molecule from the intracellular environment. On leaving the cell, the glucose molecule must present the C4/C6 end to bind the internal binding site.

The alternating conformation model explains the specificity of cytochalasin B as an inhibitor of glucose efflux, and shows why it is not transported and therefore prevents the conformational change to the outside-facing state (Taverna and Langdon, 1973 and Lin and Spudich, 1974). This also demonstrates the similar function of ATB-BMPA as a specific inhibitor of glucose influx.

Not all kinetic data that has been collected for GLUT 1 have supported the alternating conformer model of transporter function. Carruthers and Helgerson (1991) have presented results which demonstrate the simultaneous existence of two binding sites within the transporter. They used maltose (which inhibits glucose uptake by binding at or close to the sugar influx site) with cytochalasin B (which inhibits glucose uptake non-competitively by binding at or close to the glucose efflux site). They found that one, when present in the uptake media, increases the $Ki_{(app)}$ for inhibition of the other, in a

manner that was consistent with simultaneous binding of both inhibitors. However, although these data demonstrate the simultaneous existence of binding sites for cytochalasin B and maltose, it does not prove that these sites represent the glucose binding sites. It has been suggested that the cytochalasin B site and the sugar efflux (internal) binding sites may be either overlapping, or mutually exclusive (Krupka, 1971; Barnett et al 1975).

1.1.5 THE FUNCTIONAL UNIT OF GLUCOSE TRANSPORT.

There may be a simpler explanation for the apparent dichotomy between the alternating conformer model of glucose transport, and the evidence for the simultaneous existence of both internal and external glucose binding sites; the functional unit.

Before the gene had been cloned, and the protein purified, the size of the glucose transporter of human erythrocytes had been determined. Using analysis by SDS-PAGE, various sources had indicated at least five different protein sizes for the glucose transporter (Batt et al, 1976; Lienhard et al, 1977; Taverna and Langdon, 1973; and Jung and Carlson, 1975). Cuppoletti and Jung (1981) used freezing, and increasing radiation dose in the form of an electron beam, to inactivate the flux of D-glucose by the transporter (without significant increase in the flux of normally impermeant sugars). Target size analysis was used to determine the size of the glucose transporter *in situ* to be 185 kDa. Jung et al (1980) had previously reported the cytochalasin B binding protein to be 200 kDa (also by radiation inactivation and target size analysis), though at the time it was unclear whether this represented the transporter, or a functionally associated protein. Cuppoletti and Jung (1981) found both to be the transporter protein. Their data, together with SDS-PAGE analyses, suggested that the D-glucose transporter of erythrocytes was multimeric. This was consistent with kinetic data which had pointed towards the same conclusion (Lieb and Stein, 1971).

Further characterisation of the GLUT 1 protein *in situ* by radiation inactivation analysis indicated an apparent M_r of 124 kDa (Jarvis et al, 1986). Other reports have indicated molecular weights of 220 kDa (Jung et al, 1980; Cuppoletti et al, 1981). These figures are very close to the predicted sizes of dimers and tetramers of a 55 kDa protein.

Hebert and Carruthers (1991) determined the size of purified GLUT 1 by cholate solubilisation, size-exclusion chromatography, and sucrose gradient ultracentrifugation. They found the transport system to exist as 104 and 226 kDa species. This was considered to be consistent with previous findings, as evidence for the formation of both dimers, and tetramers. This was tested by the expression of chimeric transporter proteins in CHO cells endogenously expressing GLUT 1. The chimera was GLUT 1 protein, with the 29 C-terminal residues replaced by the 30 C-terminal residues of GLUT 4, identifiable by specific anti-GLUT 4 antibodies. Immunoprecipitation with the anti-GLUT 4 antibody was found to also precipitate the native GLUT 1 protein of the CHO cells (where no such co-precipitation is found in cells endogenously expressing both GLUT 1 and GLUT 4 together), thus indicating a homooligomeric structure formation.

Hebert and Carruthers (1991) proposed a model for the functional arrangement of these oligomeric structures. A simplified representation of this model is shown in figure 1.4. Briefly, they propose a dimer to have four possible states, in respect of its four binding sites (part a). Hence the possibility of internal and external glucose binding sites being simultaneously available or occupied, within a transporter unit. Both monomeric parts of a dimer are proposed to be catalytically active, and to function independently of each other. However, this independence is thought to be lost in the formation of a tetramer - a dimer of dimers. Within this structure, a dimer is thought to function as a unit. That is, each dimer now only has two possible states (shown in part b of figure 1.4). The monomers are now functionally arranged

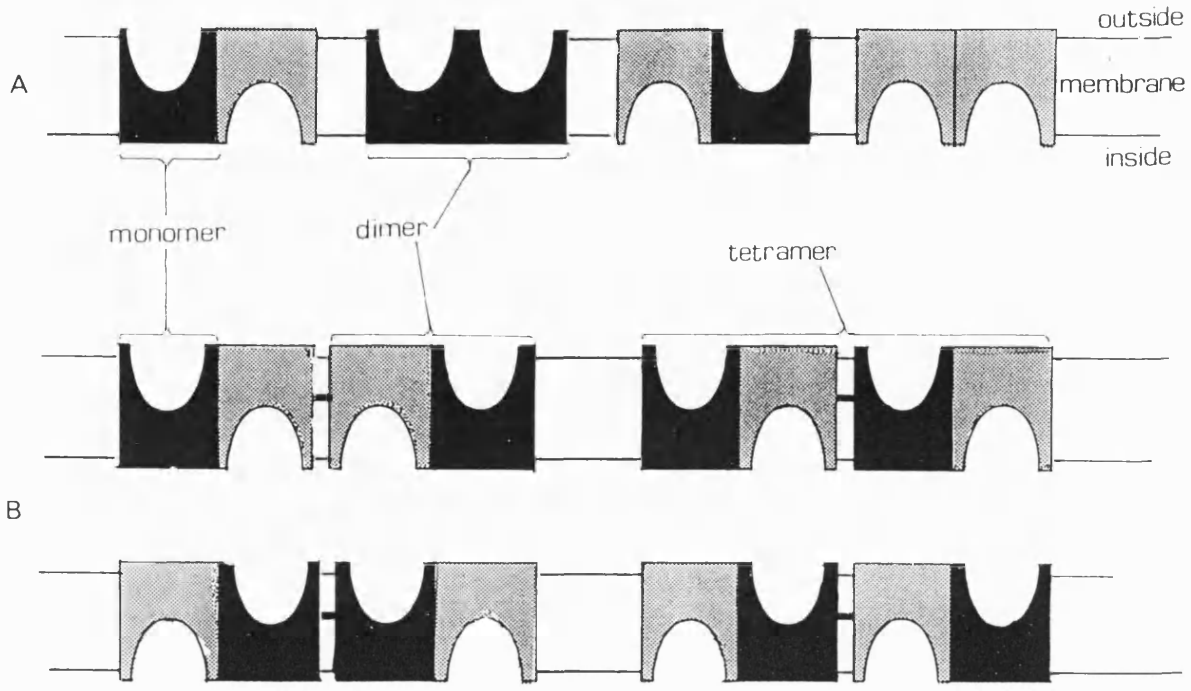


Figure 1.4 Schematic representation of the proposed functional arrangement of GLUT1 glucose transporter monomers, based on Hebert and Carruthers, 1991.

antiparallel to their partners, such that one influx and one efflux site is available per dimer. The transport of one glucose molecule by one monomer, causes a simultaneous but antiparallel conformational change in the other. In this arrangement, a tetramer always presents two influx and two efflux sites, irrespective of substrate presence, movement or direction.

1.1.6 CONTROL OF GLUCOSE TRANSPORTER FUNCTION

As has been mentioned in section 1.1.2.1, GLUT 1 appears to be regulated by circulating growth factors, hormones, and other mitogens, by inducing increases in transport activity, cell surface localisation, transcription rate, and increasing the stability of mRNA(Rollins et al, 1988; Hiraki et al, 1988). It has also been shown that reduced blood glucose levels also stimulate GLUT 1 activity (Kahn and Flier 1990; and Haspel et al,1986.), or affect expression indirectly by inhibiting its lysosomal degradation (Ortiz et al, 1992). Such influences apparently being the primary regulators of GLUT1 function, it is clear from studies of insulin- responsive tissues, that GLUT1 is also regulatable by insulin.

GLUT 4, the principle insulin-regulatable transporter isoform, is regulated by intra-cellular redistribution, as well as the more direct effects of insulin of increasing the carrier's capacity for glucose. That is, under non-insulin stimulated conditions, the GLUT 4 molecules of adipose and skeletal muscle tissue are located intracellularly, membrane bound in microsomes (Cushman and Wardzala, 1980). Insulin induces a redistribution of the GLUT 4 so that many molecules are present on the cell surface. Despite GLUT 4 being the predominant glucose transporter in insulin- sensitive tissue, insulin has also been found to cause an increase in the cell- surface appearance of GLUT 1, in a similar way (GLUT 1 cell surface localisation found to be increased by three to five- fold, where the redistribution of GLUT 4 accounts for a 15 to 20 fold increase for this isotype) (Holman et al, 1990; Clark et al,

1991). The increase in GLUT 1 cell surface availability is probably largely due to the fact that GLUT 1 is maintained at the cell surface in the absence of insulin, where GLUT 4 is virtually absent from the surface under the same conditions (Yang and Holman, 1993). This is considered to be due to a unique targeting property of GLUT 4, to the intracellular location. Levels of GLUT 1 mRNA are also maintained during glucose deprivation and during re-feeding, where GLUT 4 mRNA decreases in starvation, and rapidly increases in response to re-feeding (Sivitz et al, 1989), which also explains the difference in the magnitude of insulin and glucose -regulation between these two isotypes.

Insulin has been found to have no effect on either GLUT 1 function or distribution in non- insulin responsive tissues, where GLUT 4 is not present (e.g. brain, erythrocytes, Hep G2 cells). Although the mechanisms involved are not known, it is clear that GLUT 1 can be regulated by insulin, but only under certain, not- yet- determined conditions. Expression of GLUT 1 in CHO cells has illustrated this (Harrison et al, 1990). It was found that insulin caused an increase in 2-deoxy-D-glucose uptake in some cells expressing GLUT 1 (no increase was found in non- transfected cells). However, this increase was only found in cells expressing fairly low quantities of GLUT 1 protein. No increase was found in cells expressing high levels. It was suggested by the authors that the increase was not due to translocation of GLUT 1 to the cell surface. The principal conclusion from this work of Harrison et al would seem to be that it is specific insulin - responsive cellular processes that are important in insulin response, rather than the particular transporter isotype involved. That is, in the environment of CHO cells, the GLUT 1 protein, not usually responsive to insulin, can be made to respond to insulin, given the appropriate conditions, in this environment.

During the synthesis and membrane insertion of a glucose transporter, there is no proteolytic cleavage at any point (Maiden et al, 1987). GLUT 1

lacks a cleavable amino terminal signal sequence (Mueckler et al, 1985). Mueckler and Lodish (1986) have proposed that glucose transporters have instead, at least two distinct signal sequences within the protein sequence. They proposed the involvement of a "signal recognition particle" (SRP), an RNA component, in the targeting and membrane insertion of the transporter protein. It is proposed that such sequences, and the SRPs are responsible for the co-translational insertion of the appropriate local areas of the molecule into the membrane.

1.2 EXPRESSION OF GLUCOSE TRANSPORTERS IN A NON-NATIVE ENVIRONMENT

The use of alternative cellular environments for the expression of glucose transporters is important for a number of reasons. Firstly, if an isoform is isolated and expressed in a non- native environment, assuming the glucose transporter and transport profiles of that system have been investigated, then studies conducted can be carried out, with proper controls, on that one isoform in isolation. Mutations can be made, or the transporter protein or gene otherwise manipulated in a controlled way, and the results studied in the light of a background of the same cell type expressing either none of that transporter isotype, or the un-manipulated version. The expression of each isotype in isolation in this way has been important in the determination of kinetic parameters, and substrate specificities of the different transporters. The use of an expression system may allow simplified purification techniques, or the expression of higher levels of the protein than is available in any cell type naturally. Glucose transporters have been expressed in a number of different systems, and to different ends. Each system has its advantages and disadvantages, and the aims of the expression must be carefully born in mind in the selection of the most suitable system.

1.2.1 BACTERIAL SYSTEMS

The GLUT 1 glucose transporter, cloned from HepG2 cells, has been expressed in bacterial cells (Sarkar et al, 1988; Thorens et al, 1988). A mutant strain of *E.coli*, defective in glucose transport, was used. The transporter gene was expressed on a bacterial plasmid, and studies were made of cells harbouring the plasmid, against the mutant cells without the plasmid. Cells with the plasmid were shown to take up both D-glucose and 2-deoxy-D-glucose, where no such activity was found in the non- transformed cells. The

transport was found to be stereospecific (that is, 2-deoxy-D-glucose transport was inhibited by D-glucose, but not by L-glucose). In accordance with the normal profile of GLUT 1, transport was also found to be inhibited by cytochalasin B. The use of antibodies against specific areas of the GLUT 1 molecule, and the use of whole cells against permeabilised cells in cytochalasin B inhibition of 2-deoxyglucose transport, demonstrated that the transporter was membrane inserted, and correctly orientated. However, the limitation of bacterial systems are based on their inability to process eukaryotic proteins as they would be in a eukaryotic system. The transporter was shown to be functional, but in the absence of appropriate post- translational modification to the protein, bacterially- expressed glucose transporters would not be suitable for any structural analyses. The protein expressed in this way, when analysed by SDS-PAGE was found to have an apparent M_r of 34 kDa. In a native eukaryotic environment, the same protein has an M_r of ~55 kDa. The deglycosylated form of the protein in a eukaryotic environment is ~38 kDa. (Mueckler et al, 1985). Therefore the use of prokaryotic expression systems is valuable, but not without significant limitations.

1.2.2 CULTURED MAMMALIAN CELL SYSTEMS

The two principal eukaryotic systems which have been used for the expression of the GLUT family of glucose transporters are the mammalian cell line CHO (Chinese Hamster Ovaries), and the use of oocytes from *Xenopus laevis*.

In 1988, CHO cells were utilised to investigate the effects of altered glycosylation on the function of GLUT1 (Haspel et al, 1988). They expressed GLUT1 in wildtype (WT) CHO cells, and compared this expression to GLUT1 expressed in mutant CHO cells, deficient in N-Acetylglucosaminyltransferase I activity. Where GLUT1 expressed in WT CHOs was found to have an apparent M_r of ~55 kDa, expressed in the mutant CHO cells, this was ~ 40

kDa. The use of endoglycosidases and glycosylation inhibitors revealed a core protein of M_r 38 kDa (as native in erythrocytes) as the unglycosylated form of GLUT1 in both types of cells. This study was important in highlighting the use of this expression system to express modified forms of glucose transporters.

Harrison et al (1990) expressed GLUT1 in CHO cells in order to examine the action of insulin on the cell surface disposition of GLUT1. Expression of a long-term nature such as the CHO cell system is unpredictable, and as such, much work is involved in the production of CHO cell clones expressing the desired protein. The level of expression, per clone, is unpredictable, and not manipulable once expression is achieved. During the transfection protocol, very many cells must be used, and only a very limited number take up any DNA at all. In this way, if a transfection is a success, then often a substantial number of clones will be the result, each expressing it's own level of the foreign protein (the DNA must be stably integrated into the host cell genome, and there is no way of controlling the localisation of this integration). In this way, Harrison et al (1988) studied a number of CHO clones expressing GLUT1. They found that untransfected CHO cell native glucose transporters are responsive to insulin, showing a 40% increase in tritiated 2-deoxy-D-glucose uptake. Clones expressing only low levels of GLUT1 were also found to be responsive to insulin by a 40% increase in transport (since the presence of the GLUT1 increases the basal 2-deoxy-D-glucose uptake, this represents GLUT1 insulin responsiveness). However, in clones expressing more GLUT1, and therefore showing larger increases in basal transport rates (upto seven fold); the insulin responsiveness of the cells became lower. This work demonstrated that the action of insulin on the GLUT1 in the CHO cells was not by a translocation system. This work demonstrates the comparative value of the use of a stable expression system, when several clones can be examined.

The CHO cell system has also been used to study mutated forms of glucose transporter protein. Oka et al (1990) produced a form of GLUT1 truncated at the C-terminus by 37 amino acids (the C-terminal tail usually comprises 42). They studied glucose transport and ligand binding profiles of this mutant, in comparison to the same profiles of WT GLUT1. Such comparisons led to the conclusion that the C-terminal tail of GLUT1 is important in the conformational change required to alternate between the inward and outward facing forms of the transporter. That is, the mutant form was not able to transport glucose, and showed very limited binding of the external side-specific ligand ATB-BMPA (where the WT had shown good binding). Binding of cytochalasin B at the cytoplasmic side was at a level equivalent to that of the WT clone, and was glucose displaceable. That is, this work demonstrated that the truncation of the C-terminus resulted in a transporter which was located in the membrane, but apparently locked into an inward facing conformation.

Although these studies illustrate the merits of a stable transfection expression system, such a system is difficult to utilise, and expression levels cannot be manipulated, and are often low. For glucose transporter expression, transport studies must be conducted against a background of glucose transport from the native transporters of the cells. Although the native transporters are immunologically distinct from the expressed transporters, their function as transporters can, at best, be subtracted from observed transport values.

GLUT1, GLUT2, GLUT3 and GLUT4 at least, have now been successfully expressed in oocytes from *Xenopus* (Gould et al, 1989; Keller et al, 1989; Vera and Rosen, 1989; and Gould et al, 1991, amongst others). Perhaps the principal advantage that the oocyte system has over a stably-transfected cell line, is that the level of expression of the foreign protein can be directly manipulated. Expression is achieved by the *in vitro* production of

mRNA encoding the foreign protein, from a constructed plasmid. The mRNA is directly microinjected into the cell. The level of expression of the protein is directly dependent on the amount of mRNA injected.

Expression of cDNAs encoding GLUT1 and GLUT4 in oocytes confirmed that the cDNA actually encoded glucose transporters (Keller et al, 1989). Oocyte expression of glucose transporters has yielded much information about substrate specificities of the various isoforms (Gould et al, 1991); and comparative kinetic analyses of hexose transport by the different isoforms have been possible.

Oocytes have the advantage of being eukaryotic, and therefore posttranslational modification of expressed proteins can reasonably be expected to be true to the native forms. Oocytes do normally transport glucose, although endogenous transport activity is low. Although the oocyte glucose transport system is both kinetically and immunologically different to the mammalian glucose transporters (GLUT1 and GLUT2 studied, Vera and Rosen, 1989), the transport is stereospecific, and has been found to be inhibitable by cytochalasin B. The transport of glucose by uninjected oocytes has also been shown to be stimulated by both insulin, and insulin-like growth factor.

It is from this observation, and other work examining the expression of different glucose transporter isoforms in cells usually either responsive or unresponsive to insulin, which allowed the conclusion that the insulin response of increased glucose transport is a function of the cell in which the glucose transporters are functioning, and not a function of the isotype present.

The oocyte system is an easily manipulable transient eukaryotic expression system. It's use for the study of transport kinetics, substrate specificities and inhibition studies of the different glucose transporter isoforms has been, and will probably continue to be an important contribution to the study of glucose transporters. However, on answering the question of the three-dimensional structure of glucose transporters, the oocyte system is not

useful. Although expression levels are fully adequate for study, the expression of transporters in this way is not suitable for the production of the quantities of protein that would be required for three dimensional analysis.

Section 1.3 describes the baculovirus expression system, which, as a large-scale expression system, is likely to find its own place in the range of expression systems that have been used in the expression of glucose transporters.

The differences between the various systems that have been used for the expression of glucose transporters provide them all with a useful function. Where some are suitable for transport kinetic studies, others are more useful for the comparative analysis of various parameters of the transporter isoforms. Yet others will be useful in the large-scale production of transporters, which will become important in the elucidation of the three dimensional structure of the mammalian glucose transporter.

1.3 THE BACULOVIRUS EXPRESSION SYSTEM

Baculoviruses have been isolated only from invertebrates, with most found in insects. Baculovirus infections have been reported in over 600 species, ranging from Lepidoptera (butterflies and moths) to Coleoptera (beetles). Infection causes death, and early studies of baculoviruses were from a biological control perspective. Baculoviruses were first reported for the use of the expression of a foreign protein by Smith et al in 1983, expressing b-interferon.

The two principle advantages of the exploitation of the baculovirus as the basis of an expression system are that it is completely safe to workers, on the basis of its host range, and the fact that controlled infection permits the removal, or replacement of a gene encoding a highly expressed protein, without detriment to the infection. Other advantages of the system are that there is no practical limit on the size of the foreign DNA that can be introduced (a baculovirus genome is between 88 and 200 kbp, and the nucleocapsid expands to accommodate the DNA present). There are available strong gene promoters, allowing the expression of foreign proteins in large quantities. Since insect cells are eukaryotic, post translational processing (with the exception of glycosylation) of mammalian or other eukaryotic proteins, is usually as would occur in the normal environment of the protein.

Although a number of baculoviruses, and a range of host cells have been used as a tissue culture- based expression system, the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV): *Spodoptera frugiperda* (Sf) cell- line combination has so far been the most popular and efficient. The cell lines most commonly used are Sf21, from the IPLB-Sf21-AE cell line, derived of ovarian tissue, or the Sf9 line, a clonal expansion of Sf21 (Vaughn et al, 1977, Summers and Smith, 1987). The use of tissue culture as opposed to the use of insects is in itself an advantage of this system,

in that specialist equipment and insect rearing techniques are not required (although the baculovirus expression system can be used in whole insects).

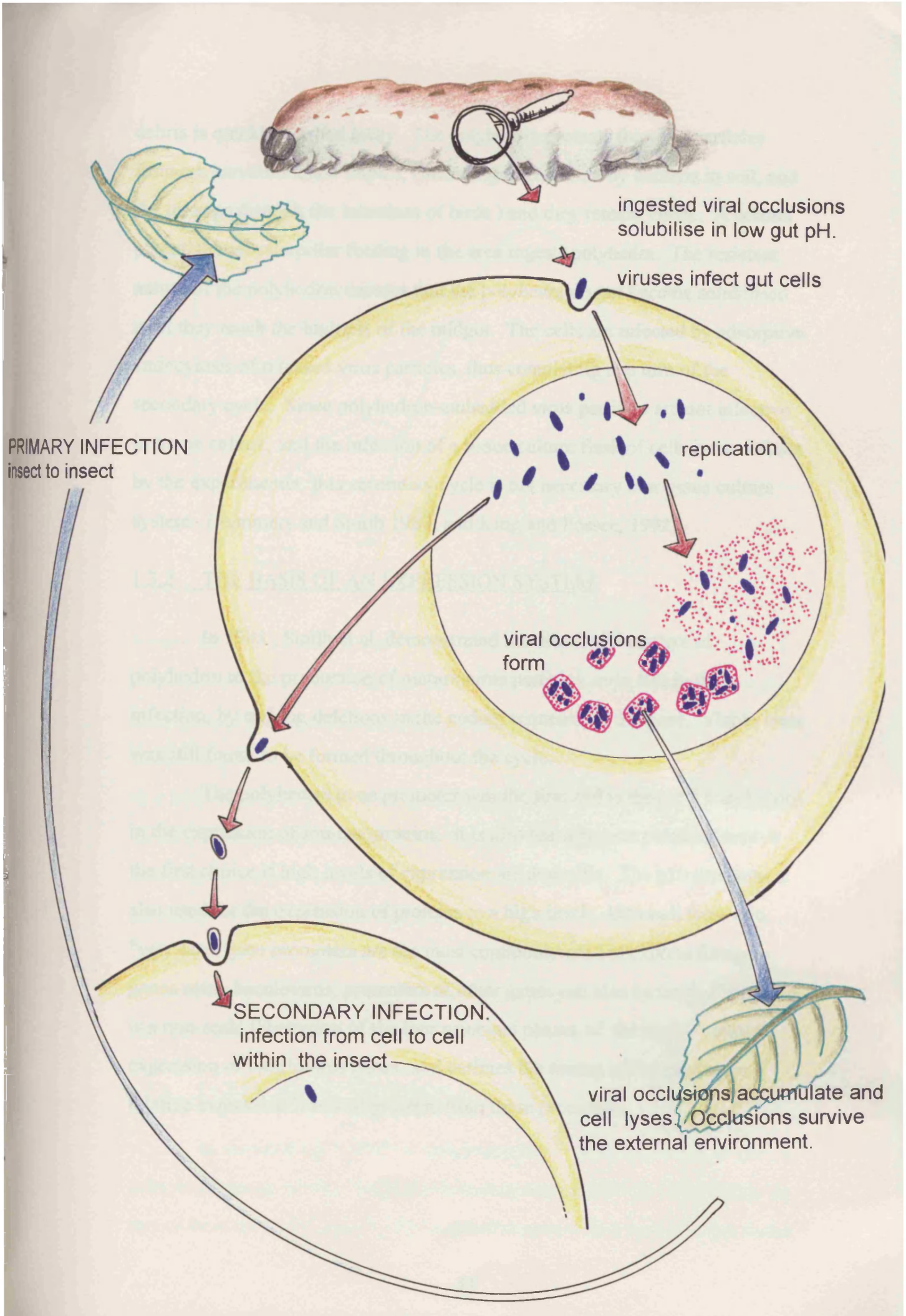
1.3.1 BACULOVIRUS LIFE CYCLE

A description of the natural life cycle of the baculovirus in the insect host is the best way to illustrate many of the features that make this combination successful as an expression system. Figure 1.5 illustrates the main features of the life cycle of AcMNPV in a fall army worm caterpillar (*Spodoptera frugiperda*).

The first cells of a new host to be infected are those of the mid gut. The virus DNA genome rapidly replicates in the nucleus of the cell, and virus nucleocapsids are produced within eight hours of the infection. By 12 hours post infection (h.p.i.), virus nucleocapsids bud out of the nucleus, and subsequently bud through the plasma membrane, acquiring a lipid envelope (which, as part of the infection cycle already contains viral proteins). These mature particles infect other cells nearby. This represents one turn of the primary cycle of a two cycle system. In this way, almost all of the cells of the insect become infected. In a tissue culture system, this represents the infection of a whole dish, flask or suspension culture of cells.

Returning to the progress in the original cell: after having released a number of mature virus particles, the production continues. Whole mature viruses, with lipid envelopes are produced within the nucleus. After about 18 h.p.i., polyhedrin, encoded by the virus, is produced in the nucleus. This is a tough, crystalline protein, into which the new virus particles become embedded, to form viral occlusions, "polyhedra". This protein, and an associated protein, p10, is produced in very large quantities, and very many polyhedra are formed in the nucleus, which swells to almost fill the cell before it finally lyses. Very soon all of the cells of the host lyse, and the insect becomes a soup of polyhedra, virus and debris. In a natural environment, the

Figure 1.5 The baculovirus lifecycle. Schematic representation of the bi-phasic lifecycle of the AcMNPV baculovirus in a *Spodoptera frugiperda* cell type. The first cycle is represented by red arrows, and the second by blue arrows.


ingested viral occlusions solubilise in low gut pH.
viruses infect gut cells
PRIMARY INFECTION
insect to insect
replication
viral occlusions form
SECONDARY INFECTION.
infection from cell to cell within the insect
viral occlusions accumulate and cell lyses. Occlusions survive the external environment.

debris is quickly washed away. The polyhedrin protects the virus particles from any environmental impact, (including degradation by bacteria in soil, and the passage through the intestines of birds) and they remain viable. A second potential host caterpillar feeding in the area ingests polyhedra. The resistant nature of the polyhedrin ensures that the polyhedra do not become solubilised until they reach the high pH of the midgut. The cells are infected by adsorptive endocytosis of released virus particles, thus completing one turn of the secondary cycle. Since polyhedron-embedded virus particles are not infective in tissue culture, and the infection of a tissue culture flask of cells is carried out by the experimenter, this secondary cycle is not necessary in a tissue culture system. (Summers and Smith 1987; and King and Possee, 1992)

1.3.2 THE BASIS OF AN EXPRESSION SYSTEM

In 1983 , Smith et al demonstrated the dispensable nature of polyhedrin to the production of mature virus particles, even late in the infection, by making deletions in the coding sequence of the gene. Viable virus was still found to be formed throughout the cycle.

The polyhedrin gene promoter was the first and is the most widely used in the expression of foreign proteins. It is also the strongest promoter, and so the first choice if high levels of expression are desirable. The p10 promoter is also used for the expression of proteins to a high level. Although these two "very late" gene promoters are the most commonly used to express foreign genes using baculovirus, promoters of other genes can also be used. Figure 1.6 is a non-scale illustration of the four principal phases of the highly ordered expression of baculovirus genes, and outlines the timing and approximate relative expression levels of proteins from these promoters.

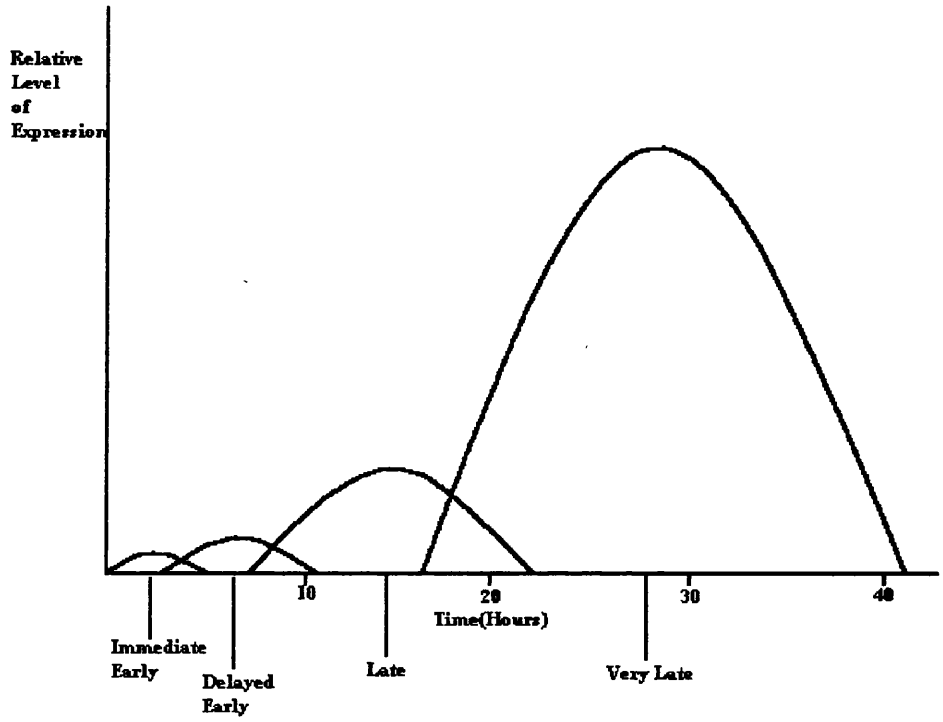


Figure 1.6 Phases of baculovirus protein expression during an infection. (Not drawn to scale - approximate comparison of expression levels of protein from the four principal phases of baculovirus gene expression only).

Non polyhedrin/p10 promoters are not used for large- scale expressions, but earlier promoters are useful if expression level is relatively unimportant but a high degree of post-translational modification to the foreign protein is necessary. Also, the baculovirus system can be used for the production of cell lines for longer-term expression of foreign protein. This thesis is concerned only with the polyhedrin gene promoter, and others will not be discussed further.

1.3.3 EXPRESSION OF FOREIGN PROTEINS

In the wildtype AcMNPV, polyhedrin is optimally produced in Sf9 cells, to represent 50% of the total cell protein content (500 to 1200 mg/L). In theory then, the replacement of the polyhedrin gene with a foreign gene, under

the same promoter, might be expected to achieve similar levels. However, although high levels are usually achieved, levels matching those of polyhedrin are rare. Examples of proteins that have been produced to high levels include the expression of LCMV N protein (lymphocytic choriomeningitis virus nucleoprotein) to represent approximetely 50% of the total cellular protein (Matsuura et al, 1987); the LCMV-N (lymphocytic choriomeningitis virus N protein),expressed to around 3μg/mg whole insect protein (Emery and Bishop, 1987), and human β-interferon expressed to $10\mu g/10^6$cells (Smith et al 1983b) However, a yield of 1mg/L is more realistic for many proteins. Unfortunately, beyond using transfer vectors which have been designed to include the features important for maximal protein expression, there is presently no reliable way to predict the level of synthesis that will be achieved by a recombinant protein, except the very general guide that the greater the post- translational modification that is required, the lower the expression may be, with membrane-associated glycoproteins usually becoming expressed to the lower end of this range. However, it has been suggested that expression levels may be improved upon by slight sequence modifications to the foreign gene, to optimise the codon usage to match the usage of insect cells (Cameron et al, 1989).

A number of other factors have also been shown to effect the final yield of a foreign protein. Licari and Bailey (1991) expressed β-galactosidase as a fusion protein, as a model to study such factors. The effects of the multiplicity of infection (moi) used to infect cells, were found to depend very much on the growth phase of the cells at the time of infection. That is, if cells were infected in the early phase of exponential growth, the final yields were found to be relatively independent of the moi used (using a range of 0 to 100 pfu/cell). However, if infection was deferred until late in the exponential phase of growth, a logarithmic relationship was found between moi and final foreign protein yield. Licari and Bailey (1991) also studied the effects of protein degradation. It was found that up to about 24 hpi (about the time the

expression of many foreign proteins begins to be detected) the rate of protein degradation was found to be of the same order of magnitude as the rate of synthesis. This may be a reason why foreign proteins are rarely detectable much earlier than this, since with the use of an extremely sensitive method of detection, foreign protein expressed in Sf9 cells has been detected as early as one hour post infection (hpi) (Karp et al, 1992). But, as synthesis increases rapidly up to around 96 hpi, the rate of degradation actually slows down, until its effects become insignificant to the net production of protein.

1.3.3.1 POST- TRANSLATIONAL MODIFICATION

Protein processing of virtually every type has now been successfully demonstrated of proteins expressed in insect cells. These include glycosylation, fatty acid acylation (myristillation and palmitoylation), nuclear transport, disulphide bond formation, C-terminal adaptation, phosphorylation, proteolytic cleavage of precursor proteins and signal sequences, and the formation of complex tertiary and quaternary structures. A number of post-transcriptional processes have also been demonstrated in insect cells, including polyadenylation and 5' capping of mRNAs. However, the recognition and appropriate splicing out of introns have so far only been hinted at (Jeang et al, 1987). Since glycosylation, and possibly the formation of tertiary structure, are the only types of processing with which the work described in this thesis is directly involved, examples of the other types of processing will not be discussed. A number of comprehensive reviews of this subject are available (King and Possee, (1992); Licari and Bailey (1991); Bishop and Possee (1990) and Possee et al (1990)).

Glycosylation

A large number of glycoproteins have been expressed using the baculovirus expression system. From these, two general rules have been determined. The protein, as most proteins expressed in this way, is usually found to be biologically active, often to the same level as the native protein in its normal host cell; and the glycosylation is usually present, in the appropriate location(s) on the protein (Cameron et al, 1989), but is of a different nature to the native protein.

Butters and Hughes (1981) studied the membranes from an established mosquito cell line. both N-linked and simple O-linked glycosylation was found. The N-linked oligosaccharides found were of the high mannose type. In mammalian cells, high mannose oligosaccharides are the starting point of N-linked glycans. However, in mammalian cells, these structures are extensively trimmed, and then "decorated" - modified to form complex branching structures by the addition of sialic acid, fucose, galactose and glucosamine-galactose residues. Butters and Hughes concluded that insect cells lack the relevant enzymes for such modification, and therefore are unable to modify the core oligosaccharide structures. That is, the oligosaccharides of insect glycoproteins are essentially the same as mammalian intermediate oligosaccharides.

Endo-H (endo-β-*N*-acetyl-D-glucosaminidase H) specifically removes immature high mannose type oligosaccharides. Most mammalian glycoproteins are therefore endo H- resistant. The demonstration that insect cell glycoproteins, and mammalian glycoproteins expressed in insect cells, are Endo- H sensitive (as determined by a reduction of apparent M_r on SDS-PAGE analysis) shows that the oligosaccharides are the high mannose type, as the same, or corresponding proteins in mammalian cells are usually endo H resistant (Hseih and Robbins, 1984). It has been shown, however, that insect cells are capable of trimming the core oligo-mannose structure, though this

study did not find evidence of modification following trimming (Kuroda et al, 1990). Lucklow and Summers (1988) also concluded that insect cells were capable of both high mannose and "hybrid" type carbohydrates, and simple O-linked chains.

In 1989, however, the established generally held rules about the nature of glycosylation in insect cells were refuted. Jarvis and Summers used human plasminogen activator as a model for the study of the insect/baculovirus glycosylation pathway. Their studies demonstrated that at least some of the N-linked glycans were endo H resistant. Davidson et al (1990) studied human plasminogen, and found 40% of the N-linked oligosaccharides to be of the complex- type. This indicates the presence of one or all of the trimmed oligosaccharide modifying enzymes previously thought to be only found in mammalian cells.

Having established what, in the way of glycosylation, insect cells are capable of, however, does not appear to allow any predictions as to the state of glycosylation that will be achieved by a glycoprotein expressed in insect cells.

Germann et al (1990) expressing the human multidrug transporter, a membrane associated glycoprotein, in insect cells, found that the apparent M_r on SDS-PAGE analysis corresponded to the size of the non-glycosylated form of the normal counterpart. Despite this however, biological activity, as determined by specific photolabelling (drug resistance not being measurable because of the lytic nature of the viral infection), was measured, but found to be much lower than for the protein expressed in a normal host cell. Vissavajjhala and Ross (1990) expressed the extracellular domain of human nerve growth factor (expression of the extracellular domain only of a usually membrane anchored protein allows secretion of the protein, allowing simplified purification procedures.). They also found an apparent lack of glycosylation, or very reduced glycosylation compared to the normal protein.

In contrast, for perhaps the majority of insect cell expressed glycoproteins, reduced glycosylation has been observed and, where studied, been found to be endo H sensitive - the high mannose type. For example, Greenfield et al (1988) expressing the EGF (epidermal growth factor) receptor, and Paul et al (1990), expressing the human insulin receptor, both observed a significant difference in apparent molecular weight between the proteins in their normal host cell environment, and their expression in insect cells. When studied further, the carbohydrate chains of these glycoproteins were found to be endo H sensitive, and therefore concluded to be of the high mannose type. Both proteins were reported to be fully biologically active. Similar molecular weight differences have been observed in many other glycoproteins, including the glycosylphosphatidylinositol- linked complement inhibiting protein (Davies et al, 1993), the human transferrin receptor (Domingo and Trowbridge, 1988), and the human β_2-adrenergic receptor (Reilander et al, 1991). All of these proteins have also been demonstrated to be biologically functional. Chen et al (1991) used the baculovirus system to express the β-subunit of human choriogonadotrophin. Of the molecular weight of this protein, in its native environment, 33% is carbohydrate. In this environment, the protein has two complex-type N-linked carbohydrate chains, and four O-linked chains. When expressed in insect cells, although the extent of the glycosylation was much reduced, it appeared that both N- and O- glycosylation sites had been recognised by the insect enzymes, and the localisation of all of the carbohydrate chains was apparently correct. The N-linked chains were found to be the high mannose type. Again, the carbohydrate difference did not effect either the immunogenicity or the biological activity of the protein, which was comparable to the activity of the native protein.

A limited number of glycoproteins that have been expressed in insect cells, have shown evidence of limited processing of the oligosaccharides. The human insulin receptor is a disulphide- linked heterotetramer of α and β

subunits, the biosynthesis of which is complex, and involves the synthesis and processing of a single polypeptide- chain precursor, to the subunits. Sissom and Ellis (1989) used the baculovirus system to express a soluble secreted extracellular binding domain of the insulin receptor. The precursor protein was detected in the cytoplasm of the insect cells. The apparent M_r was found to be consistent with a reduced level of glycosylation (which occurs at a number of sites). Treatment of the precursor protein with endo H demonstrated that all of the oligosaccharide chains were of the high mannose type. However, on processing and secretion of the precursor protein to the mature secreted form, some endo H resistance was detected. The level of resistance was consistent with the trimming of some, but not all, of the oligosaccharide chains. Further analysis confirmed that the trimmed oligosaccharides were not further processed to mature complex forms. Again, biological activity was comparable to both the normal insulin receptor, and the truncated secreted version expressed in mammalian cells.

It seems, from studies of many insect cell expressed glycoproteins, that in general, biological activity is dependent on the presence of glycosylation per se, and not on the type of oligosaccharide chains present. However, predictions of the glycosylation state and functional characteristics of any given glycoprotein are still not possible.

1.3.4 BACULOVIRUS TRANSFER VECTORS

Due to the size of the AcMNPV genome, 130 kbp, direct manipulation for cloning purposes is practically impossible. This necessitates the use of transfer vectors, encoding appropriate areas of the AcMNPV genome for later re- introduction into it by recombination. Transfer vectors are based on bacterial plasmids, containing enough of the plasmid to enable the cloning of the required gene sequence to be carried out in *E.coli.* using standard

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techniques. Figure 1.7 shows a schematic representation of the construction of a transfer vector.

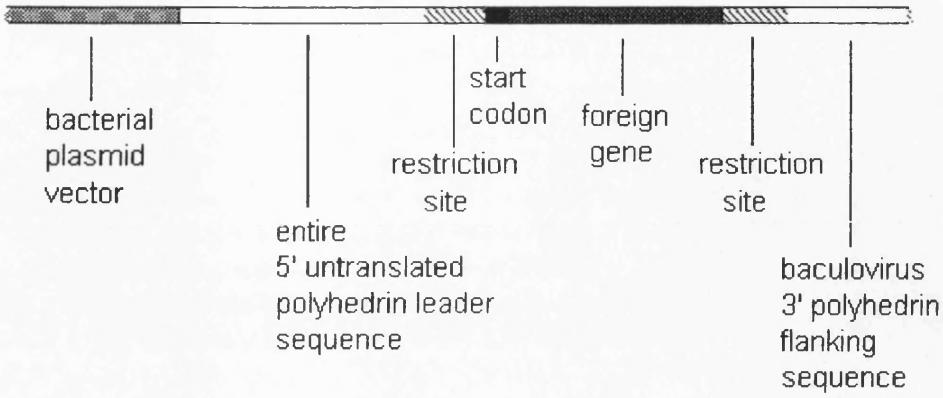


Figure 1.7 Generalised arrangement of the major components of a polyhedrin-based baculovirus transfer vector.

The other key elements in a transfer vector are (in the case of a polyhedrin- based system) the polyhedrin gene promoter, and transcription termination signals - set at either side of the cloning site for the foreign gene. The precise requirements of the sequence 5' upstream to polyhedrin for the expression of a foreign gene have been fully elucidated (Smith et al, 1983b; Matsuura et al, 1987; Possee and Howard, 1987; Possee et al, 1991) so that optimal expression is available from vectors produced. Flanking these (see figures 1.7 and 1.8) are areas of DNA corresponding to the areas normally flanking the polyhedrin gene in the AcMNPV genome. These elements are taken form an AcMNPV genome as a fragment. The polyhedrin gene is on a contiguous sequence of DNA, in the middle of a 7.3 kbp Eco R1 restriction fragment (Summers and Smith, 1987; Bishop and Possee, 1990). This excised fragment, modified to remove the polyhedrin coding sequence (Smith et al,

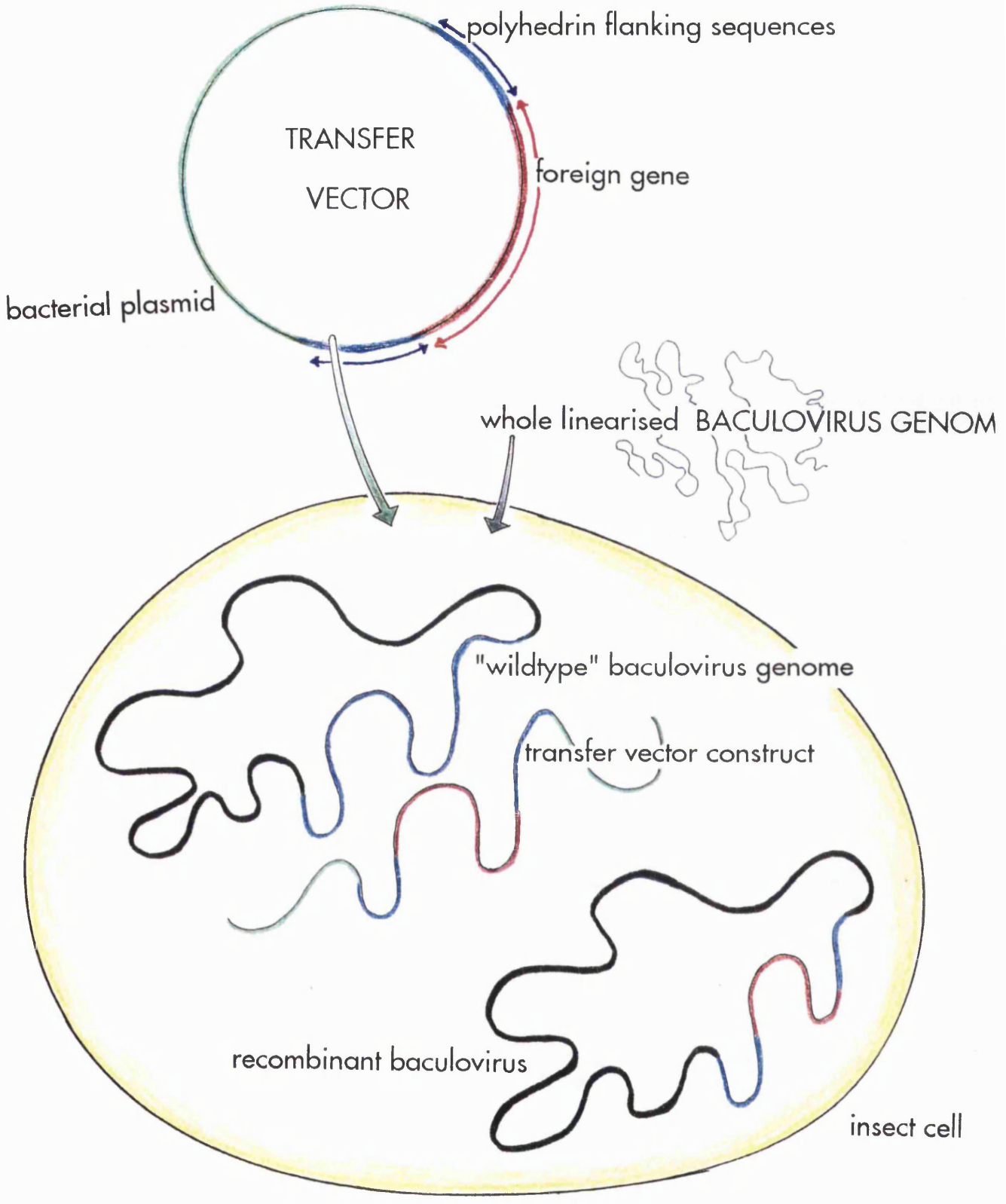


Figure 1.8 Schematic representation of the general components in the construction of a baculovirus transfer vector, co-transfection with AcMNPV genome, and homologous recombination events allowing the replacement of the polyhedrin gene with the foreign gene.

1983a; Pennock et al, 1984; Matsuura et al, 1987) has been used as the basis for many transfer vectors.

1.3.5 PRODUCTION OF RECOMBINANT BACULOVIRUSES

Figure 1.8 illustrates the principles involved in the production of a recombinant baculovirus, encoding a foreign gene in the place of polyhedrin. A transfer construct, containing the foreign gene, is co- introduced into an insect cell with whole baculovirus genome DNA (methods of co- transfection and preparation of viral genomic DNA will be discussed shortly). Homologous recombination in two areas, between the corresponding polyhedrin flanking regions (flanking polyhedrin in the genome and the foreign gene in the transfer construct) results in the replacement of the polyhedrin gene with the foreign gene. This DNA is then incorporated into a virus, infection with which will result in the corresponding production of the foreign gene product. The entire progeny of viruses, or sample of, must then be screened, in order to identify the recombinant viruses against a background of polyhedrin- encoding viruses.

In practice, however, these simple principles have been found to be fraught with problems. Principally, the number of recombinant viruses following a co- transfection with infectious AcMNPV DNA is usually expected to be at a rate of less than 1%; and the recognition of infected cells (in a plaque assay) that are not producing polyhedrin generally requires a lot of time and patience, and an experienced eye.

In recent years however, there have been a number of principal advances in the production of recombinant baculoviruses, which have resulted in the simplification of the procedure, which in turn has resulted in a reduction in the time and frustration involved. Most of the advances described have been exploited during the work described in this thesis.

The first of these (in procedure order) is the use of linearised genome DNA. In yeast and mammalian cells, DNA containing double-stranded breaks

are known to be highly recombinogenic (Bollag et al, 1989) , and the exploitation of this has become a routine method for recombination of homologous DNA sequences in such systems. This observation was tested in insect cells. Kitts et al (1990) compared infectivity and recombination frequencies of circular and linearised forms of AcMNPV. This was made possible by the introduction of a unique restriction site into the DNA (Matsuura et al, 1987). When introduced into Sf cells with a five-fold excess of plasmid DNA, linearised AcMNPV DNA was found to have only 1/150th of the infectivity of circular DNA co-transfected in a parallel experiment. Although recombination itself was only found to be ten to 12- fold higher for linear molecules than circular; the reduction in infectivity results in a reduction in the background of non-recombinant viruses. Therefore, whereas with circular virus DNA recombinant usually represent < 1% of the total progeny, for linearised virus DNA, recombinant viruses represent 16 to 39% (Matsuura et al, 1987; King and Possee, 1992).

This advance has meant that fewer progeny viruses need to be screened in order to identify recombinants, as well as having an improved number of recombinants to find.

A significant advance in the improvement of the baculovirus expression system can, co-incidentally, also incorporate the first. The single unique restriction site, Mst 11 (also called Bsu 361 and Sau 1) was found to exist within the coding region of the *E.coli* β-galactosidase (*lacZ*) gene. This gene, when introduced into the AcMNPV genome (for example, to replace the polyhedrin gene of the wildtype virus) can be used to give a blue colouration to cells infected with that virus, in the presence of X-gal (Pennock et al, 1984; Possee and Howard, 1987).

In a co-transfection, an ordinary transfer vector construction is used, and the AcMNPV.*lacZ* DNA is used in place of the normal parental wildtype (for example, as used by Pennock et al, 1984 and Possee and Howard 1987). In

non-recombinant viruses, the "wildtype" retains the blue phenotype, which can be easily detected in a plaque assay. In a recombinant virus, the *lacZ* gene is displaced by the foreign gene, and the blue phenotype therefore removed. Recombinant virus plaques can be easily detected as white (non blue). Using this approach, 40 to 80% of the white plaques in a first round plaque assay can be expected to contain the appropriate foreign gene (King and Possee, 1992). If the AcMNPV.*lacZ* DNA is linearised prior to co-transfection, then the advantages of improved proportions of recombinants to non- recombinants, and the ease of recombinant identification, are simultaneously conferred.

Since it has been reported that some workers experience problems in identifying white plaques against a background of parental- type blue plaques, the *lacZ* gene has also been utilised in another way. For example, in transfer vectors using the polyhedrin gene promoter for the expression of a single foreign gene; the p10 promoter has been used for the expression of the *lacZ* gene (i.e. a dual expression vector), which thus acts as a "reporter". Used with a normal wildtype AcMNPV (or AcMNPV.SC), recombinant plaques now appear as blue, against a background of polyhedrin containing "white" plaques (Vialard et al, 1989). The main disadvantage of this approach is that infected cells will continue to co-express β-galactosidase as well as the protein of interest.

A third significant improvement to the baculovirus system is the introduction of improved methods of co- transfection. Traditionally, co-transfection was carried out using calcium phosphate co-precipitation. This technique involves precipitation of both genome DNA and transfer vector DNA to an exactly appropriate size for entry into the cells, involving a very careful balancing of pH (Smith et al, 1983). This technique, though widely used, was not very efficient, and the results were not reproducible. Two other methods are now commonly used for the introduction of DNA into insect cells.

Electroporation involves the temporary formation of pores in the surface of the cells by subjecting them to high voltages. This has been found to be much more efficient than calcium phosphate co- precipitation, and highly reliable and reproducible. For co- transfection of wildtype AcNPV and a transfer plasmid carrying a *lac*Z gene; electroporation has been found to produce upto 100 times more recombinant viruses than are produced using calcium phosphate co- precipitation. Single transfection of AcNPV has been shown to be upto 1000 times more efficient using this method (Mann and King, 1990). The principle disadvantage of this system is that expensive highly specialised equipment is required, which can be restrictive for its use.

Cationic lipid - mediated co- transfection is now probably the method of choice when using the baculovirus expression system. The reagent involved is the cationic lipid *N*[1,1-(2,3-dioleyloxyl)propoyl]-*N,N,N,*-trimethylammonium chloride, and it is readily commercially available from a number of sources. Virus titres obtained using this method have been found to be at least 20- fold higher than those obtained using the calcium phosphate precipitation method. The percentage of recombinant viruses produced using circular AcMNPV is five to 50 times higher using this method than using calcium phosphate precipitation (Greobe et al, 1990). This method is cheaper and easier to set up in a laboratory than an electroporation system, and is much more efficient and more reliable than the calcium phosphate system.

A feature of the baculovirus system which is becoming widely exploited is the ability to simultaneously express two ore more recombinant proteins in the same cells, thus allowing the analysis of interactions forming between the expressed proteins. The formation of complexes between the SV40 large T antigen and human p53 proteins (O'Reilly and Miller, 1988), and the complex of two influenza virus polymerase proteins (St Angelo et al, 1987), as described in section 7.3.1. The use of transfer vectors capable of encoding more than one foreign gene is useful to this end. In such a vector for example,

both "very late" phase expression promoters (polyhedrin and p10) can be used, or two copies of the polyhedrin promoter may be included. As is mentioned previously in this section, a vector carrying a LacZ reporter gene encoded by the p10 promoter, and the foreign gene under the control of the polyhedrin gene is available as a colour selection system for the identification of foreign protein-expressing cells.

1.4 EXPRESSION OF GLUCOSE TRANSPORTERS IN INSECT CELLS

Since the onset of this project in 1989, the baculovirus expression system has been successfully used to express the GLUT 4 glucose transporter (Woon et al, 1991), and the full- length GLUT 1 glucose transporter (Yi et al, 1992). Because of the obvious intimate relevance of both of these studies to the work described in this thesis, greater reference is made to both in the Discussion (Chapter 7).

Although glucose transporters of mammals, and to a certain extent of bacteria and yeast, have been well characterised (see section 1.1.2), the glucose transport systems of insects have not. Wang and Wang (1993) have characterised the glucose transport system of K_c cells of *Drosophila melanogaster*, an established insect cell line. They demonstrated stereospecificity of transport, and an apparently unique substrate specificity; having greatest affinity for D- glucose, and lower, but equally efficient transport of both D-fructose, and D-galactose. Transport studies revealed that the *Drosophila* glucose transporter is inhibited by cytochalasin B. The inhibition level was found to be similar to that demonstrated by GLUT 2 (a K_i of 4mM; GLUT 2 K_i for cytochalasin B is 1 to 2 mM), which is an order of magnitude higher than for GLUT 1 (K_i of 0.1mM). Since inhibition by phloridzin, an inhibitor of transport in sodium/glucose symporters but not in the GLUT family, is almost negligible; the inhibition profile of the *Drosophila* K_c cell glucose transporter is more similar to those of the GLUT family. However, rat cDNA glucose transporter probes showed no cross- hybridisation with *Drosophila* RNA, demonstrating that, despite functional similarities, there is apparently no sequence homology between the glucose transporters of the *Drosophila* K_c cells and the mammalian facilitated glucose transporter family.

1.5 AIMS OF THE PROJECT

1.5.1 EXPRESSION OF FULL LENGTH GLUT 1 PROTEIN.

At the onset of this project, no glucose transporter isoform had been expressed in insect cells using the baculovirus expression system.

Although the primary structures of the mammalian facultative glucose transporter family have been elucidated, and the two dimensional topology has been mapped, little is really known about the three dimensional structures of these molecules. The two principal reasons for this are firstly that they are very closely membrane associated proteins, presenting problems with the purification of structurally and functionally intact molecules. Secondly, no tissues express any isoform in sufficient abundance to realistically allow the purification of sufficient quantities of protein for three dimensional structure determination.

The baculovirus expression system is known to be a large scale expression system, and has been found to be capable of the production, using reasonable resources, of milligram quantities of some proteins (see section 1.3.3). It was on this basis that the baculovirus system was chosen for the expression of the erythrocyte type glucose transporter, GLUT 1. Although purification of any expressed protein would be beyond the scope of this project, the principal aims of the expression of GLUT 1 in this way were to determine whether indeed it could be expressed to a high level, and to determine whether the protein would be biologically functional within the insect cells. That is, if the protein could be expressed to high levels, if no functionality could be shown, it would not be a suitable substrate for further research. Biological function would provide an indication that the expressed protein had adopted a functionally appropriate conformation within the host insect cells.

1.5.2 INDEPENDENT EXPRESSION OF THE TWO HALVES OF THE GLUT 1 PROTEIN.

GLUT 1 was the first of the mammalian family of glucose transporters to be identified, and as such is probably the best characterised to date. Its mode of action, functional structural unit, substrate specificity, and the localisation of the glucose binding sites to as close as a few amino acid residues, have all been well investigated (see sections 1.1.3 to 1.1.5). Most work on the sites of glucose binding and transport have indicated that the two ligand binding sites (one internal and one external), and possibly also the transmembrane channel all reside within the realms of the C-terminal half of the molecule (figure 1.1 depicts the proposed two dimensional topology of the GLUT 1 molecule, and shows the proposed ligand binding sites. Section 1.1.4 describes some of the work providing evidence for this). Based on this, and on the basis that the full length transporter would be found to be functional, it was proposed that expressed alone, the C-terminal half of the protein would be functional. Evidence that at least one signal sequence for membrane insertion exists on the C-terminal one third of the protein (Mueckler and Lodish, 1986) suggest that membrane localisation may be achieved if the insect cells recognise the signal sequence. To mirror this, it would be expected that the N-terminal half similarly expressed would not be shown to be functional, but, having also at least one signal recognition site (Mueckler and Lodish, 1986), would be expected to be inserted into the membrane.

The dual expression of both "proteins" in the same cells was based on results obtained from functional analyses of the individually expressed half "proteins", particularly the C-terminal half. The rationale for this work is discussed fully in chapter 6 ("Dual Expression").

CHAPTER 2.

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICAL REAGENTS

Tryptone and yeast extract were from Oxoid Ltd., London, UK.

Agar was from Difco Labs, Detroit, Michigan, USA.

Redistilled phenol was from Rathburn Chemicals Ltd., Walkerburn, UK.

N,N,N',N'-tetramethylethylene diamine (TEMED), sodium dodecyl sulphate (SDS), 2-mercaptoethanol, ammonium persulphate, and acrylamide were from BDH Ltd., Poole, UK.

Caesium chloride was from Rose Chemicals, London, UK.

Deoxy-nucleotide triphosphates were from Pharmacia, Upsala, Sweden.

Polyallomer quick seal ultracentrifuge tubes were from Beckman Instruments Inc., Palo Alto, USA.

5-bromo-4-chloro-3-indolyl-β-D-thiogalactoside (X-gal), and isopropyl-β-D-thiogalactosidase (IPTG) were from Northumbria Biochemicals Ltd, Cramlington, UK.

Agarose, and Low melting point agarose were from Sigma, Poole, UK.

X-ray film was from Fuji Photo Film Co. Ltd., Japan.

[γ-^{32}P] ATP was from ICN, ICN Flow, Richmansworth, Herts, UK.

2-Deoxy-D[2,6^{3}H] glucose (52Ci/mmol), and [U-^{14}C] sucrose (aqueous solution at 23GBq/mmol, ^{125}I-protein A, (0.1μCi/ml), [2(n)-^{3}H]-Cytochalasin B, and Cytochalasin E were from Amersham International PLC, Aylesbury, UK.

ATB-[-2-^{3}H]-BMPA photoprobe (activity 10 Ci/mmol) was made at the University of Bath, by Dr G D Holman, using the method as described by Clark and Holman, 1990.

Molecular weight markers and prestained molecular weight markers for SDS PAGE were from Bio-Rad Laboratories, 3300 Regatta Boulevard, Richmond, CA 94804

Nonaethylene glycol dodecyl ether (Thesit) was from Boehringer Mannheim.

Protein A sepharose was from Sigma.

ECL Western blotting kit, Amersham, Upsala, Sweden.

All other chemicals were from Sigma, Poole, UK; BDH, Poole, UK; and Fisons, Loughborough, UK.

Laboratory grade solvents were from BDH and Fisons.

2.1.2 ENZYMES, VECTORS AND PLASMIDS.

Restriction enzymes, T4 DNA ligase, λ DNA for restriction digestion for use as DNA standard markers on agarose gel electrophoresis, and T4 polynucleotide kinase were from Northumbria Biochemicals, Cramlington, Northumberland, UK.

Restriction enzymes also from Pharmacia LKB Biotechnology, Central Milton Keynes, UK.

Taq DNA Polymerase (Amplitaq) was from Perkin Elmer Cetus, Valterstetten, Germany.

Gene Clean DNA purification kit was from BIO 101, from Stratech Scientific Ltd., 61- 63 Dudley St., Luton, LU2 ONP

pT7blue vector kit was from Novogen, AMS Biotechnology UK Ltd, 5, Thorney Park, Witney, Oxon, OX8 7GE.

pVL941, pVL1392 and pVL1393 baculovirus transfer vectors were from existing stocks.

2.1.3 ANTIBODIES

"anti C-terminal antibody" used throughout for the detection of Glut 1 protein was a rabbit antiserum raised against a synthetic peptide corresponding to residues 480 to 492 at the C-terminal tail of the molecule. This antiserum was kindly provided by Dr. Avril Clark (Bath University, Bath)

"anti N-terminal antibody" used throughout for the detection of Glut 1 protien, particularly the N-terminal half, was a rabbit antiserum raised against a synthetic peptide to residues 240 to 255 of the central loop region of the glut 1 molecule (see figure 1.1, section 1.3). This antiserum was kindly provided by Dr. Stephen Baldwin (Leeds University, Leeds).

2.1.4 CDNA

The cDNA used for the production of all of the glut 1 constructs was a rabbit glut 1 cDNA. This was a kindly provided by Dr. Yoshitomo Oka (Tokyo, Japan).

2.1.5 TISSUE CULTURE AND SPECIAL EQUIPMENT

Unless otherwise stated, all tissue culture disposables, media, and supplements were obtained from Gibco (Life Technologies Ltd. Cowley Mill Trading Estate, Longbridge Way, Uxbridge, UK.) Flow, (ICN Flow, 102 Witmer Road, Morsham, P.A, USA 19044 228), and Nunc Inc., 2000 North Arora Road, Naperville, Illinois, 60566.

Techne biological stirrer Spinner culture flasks were from Fisons.

Electrophoretic transfer unit used was the Multiphor II Nova Blot unit from Pharmacia LKB Biotechnology.

The photolabelling reactions were carried out using a Rayonett RPR-100 photoreactor containing RPR-3000 lamps.

2.2 METHODS

2.2.1 MOLECULAR BIOLOGY METHODS

2.2.1.1 GROWTH AND MAINTENANCE OF BACTERIA

The *E.Coli* strain TG1 : (Sup E hsd Δ5 thi Δ (lac - pro AB) F' (tra D36 pro AB+ lac Iq lac Z Δ M15)) was used throughout.

E.Coli cultures were grown in YT broth (10g yeast extract, 10g yeast tryptone, and 5g sodium chloride per litre), and sub-cultured monthly on minimal agar plates (15g bacto agar, 10.5g K_2HPO_4, 4.5g KH_2PO_4, 1g $(NH_4)_2SO_4$, 0.5g sodium citrate, 0.2g $MgSO_4.7H_2O$, 2g glucose, and 5μg thiamine HCl; per litre).

2.2.1.2 RESTRICTION DIGESTION OF DNA

Restriction digest reactions were usually carried out using manufacturers' supplied buffers, recommended reaction temperatures and incubation times (normally 37°C for 60 to 90 minutes). Restriction digestions carried out in preparation for a ligation reaction were carried out at 37°C (unless otherwise stated for a particular enzyme) overnight. Single, or concomitant double digestion reactions were stopped by the addition of EDTA to a final concentration of 20mM. The first enzyme of sequential digestions (dictated by different buffer requirements) was inactivated by heating to 85°C for 20 minutes.

Digestions for DNA analysis were not processed further before visualisation by agarose gel electrophoresis.

Digests in preparation for ligation reactions were either gel purified and cleaned by "Gene Clean" to isolate the required fragment; or extracted by phenol:chloroform , and precipitated with ethanol , to remove contaminating

fragments before use. Such processed fragments were analysed by electrophoresis to determine the success of the digestion and the purification , before use in a ligation reaction.

2.2.1.3 PHENOL: CHLOROFORM EXTRACTION OF DNA

An equal volume of phenol (pre-equilibrated to <pH 8) was added to the DNA solution, vortexed, and the two layers separated by centrifugation (6000rpm , 2 minutes). The (upper) aqueous layer was removed to a fresh tube. An equal volume of pre-equilibrated phenol:chloroform (1:1 ; pH 7.4) was added to the tube, and the extraction procedure repeated (vortexing, centrifugation, removal of aqueous phase to a clean tube). An equal volume of chloroform was then added to the aqueous phase, and the extraction procedure repeated again.

2.2.14 DNA PRECIPITATION USING ETHANOL

Unless otherwise stated, three volumes of absolute ethanol (at -20° C), and 0.1 volume 3M sodium acetate (pH 4.0) were added to the DNA solution, and mixed thoroughly. This was incubated for 2 to 16 hours at -20°C, and centrifuged (22 000g for 30 minutes) to collect the precipitated DNA. The DNA pellet was then washed in 70% ethanol , dried under vacuum in a Savant Speed Vac, and resuspended in ddH_2O (double distilled water), unless otherwise stated.

2.2.1.5 OLIGONUCLEOTIDE PREPARATION

Oligonucleotides were synthesised by the phosphoamidite method, on an Applied Biosystems 381 A DNA synthesiser. The completed oligonucleotide was removed from the synthesis column by slowly drawing through 1ml concentrated ammonia solution. This solution was then heated to 55°C for six hours, and the oligonucleotide dried down under vacuum, using a

Savant Speed Vac. The dry oligonucleotide was ethanol precipitated (section 2.2.1.4), and dissolved in 1ml double distilled water (ddH_20).

The yield of the synthesis was determined by a spectrophotometric scan of a diluted solution, from 350nm to 240nm. The concentration was calculated from the 260nm peak, assuming that 1.0 O.D ml corresponds to 37μg/ml DNA.

2.2.1.6 OLIGONUCLEOTIDE QUALITY TESTING

The oligonucleotide quality was tested by radiolabelling with [γ-^{32}P]-ATP using polynucleotide kinase, and sizing of the radio-labelled material using polyacrylamide gel electrophoresis. The reaction was carried out on 0.2 pmoles of oligonucleotide, using 0.15 pmoles gamma labelled ^{32}P- ATP, and two units of polynucleotide kinase; at 37°C for 30 minutes. A 0.3mm thick 20% polyacrylamide gel was prepared, the samples loaded , and run for 2 hours at 800 V. The gel was then visualised by autoradiography.

2.2.1.7 POLYMERASE CHAIN REACTION

The PCR oligonucleotides, prepared as above (2.2.1.5) were designed in pairs to areas of glut1 cDNA flanking the area targeted for amplification. This included slight mismatches in order to introduce unique restriction recognition sites into the ends of the amplified fragments. The reaction mixture contained 1pg template DNA (rabbit glut1 cDNA); 0.2μM of each oligonucleotide primer; 1.5mM $MgCl_2$; 50mM KCl; 10mM Tris-HCl pH 8.3; and 50μM of each dATP, dCTP, dGTP, and used 0.5 U Taq DNA polymerase (Amplitaq).

The reaction mixture (100μl) was overlaid with mineral oil, and amplified for 30 cycles (60 seconds melting at 95°C; 60 seconds annealing at 55°C; and 90 seconds extension at 72°C) in a Perkin Elmer Cetus DNA Thermal Cycler.

2.2.1.8 AGAROSE GEL ELECTROPHORESIS

DNA was electrophoresed in 1% agarose gels, with 1x Tris-borate buffer (90mM Tris, 83mM boric acid, 1mM disodium EDTA, pH 8.3), unless otherwise stated. Samples were loaded in loading dye (15% w/v Ficol 400, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol), and electrophoresed at 50 V/cm (50mA). Bands were visualised using 1μg/ml ethidium bromide and an ultraviolet transilluminator.

2.2.1.9 GEL PURIFICATION OF DNA FRAGMENTS

DNA for gel purification was subjected to appropriate restriction digestion, before being run on an agarose gel (using 1% low melting point agarose; and 1x Tris Acetate buffer (0.04M Tris-acetate; 0.001M EDTA; pH 8.0)). The gel was run until the appropriate band, visualised against molecular weight markers, using UV. light, was near to the end of the gel. The appropriate band was closely excised using a razor blade, to a microcentrifuge tube.

The agarose pieces containing the required DNA was then either heated to 55°C (until the agarose had melted) and subjected to a full phenol:chloroform extraction followed by an overnight ethanol precipitation, or purified using the "Gene Clean" kit. The manufacturers' protocol was followed, which briefly involves the addition of three volumes of NaI ,and heating to 50°C until the agarose had melted. 10μl of the provided glass milk was added to the tube, mixed, and incubated at room temperature for five minutes. The mixture was then centrifuged briefly and the pellet washed three times according to the protocol. The DNA was finally dissolved at 50°C in ddH_2O and removed from the glass milk, after centrifugation, to a clean tube. The purity of the fragment was determined by analysing 1μl of the DNA solution on a 1% agarose gel.

2.2.1.10 PRODUCTION OF "BLUNT" ENDED DNA FRAGMENTS

Purified DNA (1 to 2μg)(see section 2.2.1.9) was added to a microcentrifuge tube of mixed dNTPs at 2mM each, and 5 to 10 U of DNA Polymerase I Klenow fragment, in a volume of 50μl, including 1x buffer (supplied with enzyme), and incubated at 37°C for 30 minutes. The reaction was stopped by the addition of EDTA to a final 20mM. The DNA was then purified as described in section 2.2.1.9 before use in ligation reactions.

2.2.1.11 LIGATION OF DNA

The insert and vector DNA (molar ratio 1:2) were incubated in 25mM Tris-HCl (pH 7.5), 10mM DTT, and 4mM ATP, with 0.1 U of T4 DNA ligase for "sticky" ended ligations or 1 U for blunt ended fragments, for four hours at room temperature (sticky ends) or at 16°C overnight (blunt ended reactions). The reaction was stopped by the addition of EDTA to a final concentration of 20mM.

2.2.1.12 COMPETENT CELLS

1ml of a 10ml overnight culture of TG1 cells in YT broth was inoculated into 100ml of YT broth, and grown at 37°C with vigorous shaking, until the culture reached an OD. of 0.4 (measured at 600nm) indicating log phase growth. The cells were cooled on ice, and pelleted at 3000rpm for 5 minutes at 4°C. The cells were then resuspended in 40 ml ice cold 50mM $CaCl_2$, and incubated on ice for 20 minutes. The cells were re-pelleted, as before , and resuspended in 4ml cold $CaCl_2$. The competent cells were stored on ice for at least one hour before use.

2.2.1.13 TRANSFORMATION REACTIONS

The ligation reaction was diluted with 200µl ddH_2O, mixed with 0.3ml of competent TG1 cells in a chilled microcentrifuge tube, and incubated on ice for 45 minutes. The reaction mixture was then subject to a 1 minute 'heat shock' incubation at 50°C, followed by rapid cooling on ice. 1ml of YT broth was added and the mixture incubated at 37°C for 60 to 90 minutes. 140µl (1/10th reaction mixture) aliquots were plated onto YT agar plates (1.5% agar, containing 100µg/ml ampicillin). Where appropriate (e.g. for cloning and subcloning into pUC 18) colour selection was used (30µl of 2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) and 10µl of 0.1M IPTG (isopropyl-β-D-thiogalactoside) being added to the plates, with the cells.) The plates were then incubated at 37°C overnight.

2.2.1.14 SMALL SCALE PLASMID DNA PREPARATION

A single colony was transferred into 3mls YT broth, containing 100µg/ml ampicillin, and grown overnight at 37°C with vigorous shaking. 1.5ml of the culture was transferred to a microcentrifuge tube, and pelleted by centrifugation at 12000g for 30 seconds, at 4°C. The pellet was resuspended in 100µl ice cold TGE buffer (25mM Tris-HCl, pH 8 ; 10mM EDTA, pH 8; 50mM glucose) by vortexing. 200µl freshly prepared 0.2M NaOH; 1% SDS was added to the tube, and mixed gently. 150µl ice cold potassium acetate (5M; pH 4.8) was added, and gently but thoroughly vortexed. The mixture was centrifuged for two minutes at 12000g, and the supernatant transferred to a clean tube, where it was subject to a phenol:chloroform extraction (section 2.2.1.3). This was then precipitated with two volumes of ethanol at room temperature. The DNA was pelleted (5 minutes at 12000g), rinsed with 70% ethanol, and dried under vacuum. The dry DNA was resuspended in 40µl TE buffer (100mM Tris-HCl, pH 8; 1mM EDTA), containing 20µg/ml DNAse-

free pancreatic RNAse. This method follows that given in Sambrooke et al 1989.

2.2.1.15 LARGE SCALE PLASMID PREPARATION

A 10ml overnight culture of TG1 cells containing the appropriate plasmid was used to inoculate a 500ml culture in YT broth, containing 100μg/ml ampicillin. This culture was grown overnight at 37°C with vigorous shaking. The cells were pelleted by centrifugation (5000rpm, 10 minutes, at 4° C), and resuspended in 100ml TE buffer (10mM Tris pH 7.5, 1mM EDTA). The cells were re-pelleted (5000rpm, 10 minutes) and resuspended in 25ml of TGE buffer (10mM Tris pH 7.5, 1mM EDTA, 50mM glucose). 50ml of fresh 0.2M NaOH, 1% SDS was added, and the tubes incubated on ice for 15 minutes. 25ml 3M potassium acetate pH 4.8 were added, and the incubation continued for a further 30 minutes. The mixture was centrifuged, as before (5000rpm, 10 minutes), and the supernatant was filtered through muslin. 70ml 2-propanol was added to the supernatant, and the mixture centrifuged as before. The pellet was air dried briefly, and resuspended in 7.5ml TE buffer. 8.5g CsCl was dissolved into it , and 0.8ml 10mg/ml ethidium bromide was added. The solution was transferred to quick seal ultracentrifuge tubes, balanced, and sealed. The preparation was centrifuged in a VTi65 rotor, at 40 000rpm for 14 to 20 hours, at 15°C, using a Beckman ultracentrifuge (model L5-65).

The plasmid bands were removed from the sealed tube using a 19G needle, and 1ml syringe. The DNA was extracted with isoamyl alcohol, until all traces of ethidium bromide were removed. The resulting solution was dialysed against TE buffer to remove CsCl, overnight, with three changes of buffer. The DNA was ethanol precipitated (section 2.2.1.4), and the pellet dissolved in 1ml ddH_2O. The DNA concentration and purity were determined spectroscopically, at 260 and 280nm.

2.2.2 BACULOVIRUS METHODS

Many of the methods described in section 2.2.2 follow those of King and Possee 1992.

2.2.2.1 ROUTINE SUB - CULTURE OF Sf9 CELLS

Stocks of Sf9 cells, of passage numbers 20 to 70 were routinely sub-cultured in 25cm^2 tissue culture flasks. The monolayer was examined using a reverse-phase microscope to ensure that the cells appeared to be healthy (well rounded cells with smooth, even edges, adhering loosely to the plastic), and not contaminated with fungus or bacteria , or infected with baculovirus (cells infected with baculovirus have a distinctly crenellated, uneven circumference, and distorted appearance). The medium was removed, and the cells were removed from the plastic by gently squirting fresh TC100 (5% FCS) medium over the growing surface. One tenth of that volume was then removed to a new flask, containing 4.5ml of fresh TC100 (5% FCS) medium, and incubated at 28 °C until the monolayer was confluent. Sub-culturing was usually required every five to seven days.

Cells required for experimental work were taken from these stocks as required. If many cells were to be required, an appropriate number of 75cm^2 flasks would be used, where cells would be passaged once, to boost cell numbers before use. Very large numbers of cells were generated by using a suspension culture.

2.2.2.2 SPINNER CULTURE OF Sf9 CELLS

Cells were not routinely cultured in suspension ("Spinner") culture, but such a culture was set up as required, particularly for the production of virus stocks.

To initiate a Spinner culture, cells and medium (TC100/5% FCS), to a total volume of 50ml were added to a sterile 250ml round, flat-bottomed glass flask containing a sterile magnetic stirring bar. The cells, from a monolayer culture, were added at a density of 5 x 10^5 cells/ml. The culture was incubated at 28°C, with the flask suspended slightly above a magnetic stirrer, and the culture was stirred at a constant 50 to 70 rpm.

The cell density was monitored daily (by removal of a small sample and counting the cells using a haemocytometer). On reaching 2 to 3 x 10^6cells/ml, the culture was 'passaged'. That is, excess cells were removed, and fresh medium added to give a density of 1 to 2 x 10^5cells/ml. The culture was thus passaged at least once before cells were taken for experimental work, or increased to a larger volume; to allow the cells to adapt to spinner conditions.

If a larger number of cells were required, cells from a 50ml spinner culture, and medium, were added to a 500ml "Techne" tissue culture flask, at an initial density of 1 to 2 x 10^5cells/ml. Cells were passaged, as before, or removed or used as required. A volume of 250ml was found to be sufficient for all applications described in this thesis.

2.2.2.3 LONG TERM STORAGE OF Sf9 CELLS

Stocks of cells were held in liquid nitrogen. These were relatively low passage number cells (20 to 30), used to periodically replace the "running stocks" of cells as the passage number increased (60 to 70).

Cells to be frozen were microscopically analysed, using a reverse phase microscope, to determine whether their viability was 90% or more. Cells found to be of lower viability were not used for long term storage. Cells were removed from the monolayer by squirting with fresh ice cold TC100 (10% FCS) medium, and the density determined (counting a sample using a haemocytometer). The density was adjusted to 1 x 10^6 cells/ml. One tenth volume of tissue culture DMSO (sterile) was added, and 1ml aliquots were

transferred to cryogenic vials, and chilled on ice. The vials were then placed in the vapour-phase of a liquid nitrogen container overnight. Vials were quickly transferred to the liquid nitrogen for storage.

To retrieve cells, a 25cm^2 flask, containing 5ml TC100 (10% FCS), was pre-warmed to 28°C. The vial was removed from storage, and thawed as rapidly as possible by holding the vial, without submersion, in a clean water bath at 37°C until only a sliver of ice remained. Ensuring sterility, the contents of the vial were transferred to the flask, and incubated at 28°C for four to five hours. After this time, the medium was replaced with fresh TC100 (10% FCS). The medium was replaced again after a further 10 to 20 hours. On reaching confluence several days later, the culture was sub-cultured as normal, in TC100 (5% FCS).

2.2.2.4 CO-TRANSFECTION OF VIRUS DNA AND TRANSFER VECTOR

1μg transfer vector DNA and 200ng linearised AcNPV:LacZ "wildtype" baculovirus purified genome DNA were very gently mixed in a microcentrifuge tube. A 2:1 Lipofectin reagent : sterile water mixture, to the same volume as the DNA mixture, was put into a sterile polystyrene universal bottle. The DNA mixture was carefully added to it, and incubated at room temperature for 15 minutes. A 35mm dish had been seeded with 1x 10^6 cells the day before, and was washed twice with 1ml serum-free TC100. 1ml serum-free TC100 was added to the cells. The DNA-Lipofectin mixture was then pipetted onto the cells, the dish swirled to mix, and incubated at 28°C overnight. 1ml TC100 (5% FCS) was added to the dish. After 48 hours from the co-transfection, the medium (containing released virus particles) was "harvested" to a bijou bottle. 1ml TC100 (5% FCS), containing 15μl X-gal (2%w/v in dimethylformamide, stored at -20°C), was added to the cells. After 24 hours, the dish was examined for blue colouration of cells and/or medium, to determine whether the co-transfection had been successful.

2.2.2.5 RECOMBINANT VIRUS SCREENING BY PLAQUE ASSAY

Using serial dilutions (made in TC100/5% FCS) of the co-transfection medium, in triplicate (from undiluted "neat", to 10^{-3}), 35mm dishes of cells (seeded at $1x10^6$ cells/dish 18 hours earlier) were inoculated (100µl/dish). A negative control, using TC100 (5% FCS) only was included. The dishes were incubated for 1 hour at room temperature. An overlay, (comprising in equal volumes, freshly autoclaved 2% w/v Sea Plaque agarose in water, and TC100 (5% FCS)) was prepared. This was incubated at 37°C until required. The inocula removed, 2mls of overlay were carefully pipetted into each dish. When this had solidified, 1ml TC100 (5% FCS) was added over the overlay and the dishes were incubated at 28°C in humidified conditions for five days.

To analyse the plaque assay, X-gal and neutral red stain were used in order to visualise all plaques. Recombinant plaques were colourless, whereas non-recombinant plaques produced a blue colour on incubation with X-gal.

The liquid medium was removed from the overlay, and replaced with 1ml TC100(5% FCS) containing 12µl X-gal (made up as described 3.2.4). This was incubated at 28°C for three hours, after which 1ml neutral red stain (stock 0.5% w/v in water, filter sterilised, diluted 1 in 20 with PBS before use) was added, and the incubation continued for a further two to three hours. The liquid stain was then removed, and the dishes drained and inverted, in the dark, for at least two hours (or overnight, if plaques not clear).

Plaques were identified, and putative recombinants microscopically examined to ensure that there were no blue cells present. At least six, well isolated white plaques (putative recombinants) were selected for purification for further analysis.

2.2.2.6 PLAQUE PURIFICATION

The selected plaques were "picked" by removing a plug of agarose overlay from a plaque, containing virus particles, using a sterile glass Pasteur pipette, into a sterile bijou bottle containing 5ml medium (TC100/5% FCS). This was vortexed to disperse virus particles from the agarose plug into the medium. These "plaque-picks" were stored at 4°C.

Twelve 35mm dishes of cells (seeded at 1x 10^6/dish one day earlier) were prepared for each plaque purification, plus two dishes as controls (to be "inoculated" with medium alone). Serial dilutions were made of each plaque pick (including "neat", 10^{-1}, 10^{-2}, and 10^{-3}, diluted in TC100/5% FCS), and 100 µl of each used to inoculate dishes of cells (three dishes per dilution).

Following a five day incubation at 28°C (under humidified conditions) the dishes of cells were stained with X-gal and neutral red stain, as described in section 2.2.2.5. The plaques were inspected, to identify any contamination with "wildtype" virus (identified by any blue colouration of cells or overlay). Well isolated white plaques were picked (one from each initial plaque pick assay set) to ensure a single phenotype; and subjected to another round of plaque assay if contamination was detected or suspected.

2.2.2.7 PRODUCTION OF SMALL VIRUS STOCKS

Because the titre of recombinant viruses was generally found to be very low by this stage (as determined by titration plaque assay, section 2.2.2.9), production of small "passage 1" and "passage 2" stocks was necessary before further analysis could be undertaken.

25cm^2 tissue culture flasks were prepared one day before use, with 1 x 10^6 cells/flask. The medium was removed, and the cells inoculated with 400 µl plaque-pick inoculum for one hour at room temperature. Once the inocula had been replaced by 4ml TC100/5% FCS per flask, the flasks were incubated

at 28°C for up to seven days. Cells were inspected daily. If most of the cells showed signs of virus infection (ill-defined, highly crenellated edges, cells beginning to detach from the plastic) by three or four days post infection, the medium was harvested to a bijou bottle. Such infection indicates a sufficiently high titre for analysis of recombinant virus and/or product, and these stocks were not further passaged before analysis. If only a small proportion of the cells showed evidence of infection by this time, the medium was harvested at seven days post infection, and 1ml of this used to inoculate a fresh flask of cells for the production of passage 2 virus for analysis.

2.2.2.9 TITRATION OF VIRUS STOCKS BY PLAQUE ASSAY

Determination of virus titre (plaque forming units, pfu per ml) was carried out by plaque assay, using the basic assay protocol as described in section 2.2.2.5, with minor modifications. The dilutions required to assay a virus stock would usually be serial 10-fold from 10^{-3} to 10^{-7}, made in TC100/5% FCS, performed in duplicate, using 100µl inoculum/dish. Assay dishes were only stained with neutral red stain, since the purity of the virus had been determined.

The stained cells were analysed, and the single dilution showing the maximum number of individual plaques it was possible to count, was used for the titre determination. An acceptable titre for a large "high titre" stock was considered to be >2 to 3 x 10^7.

2.2.2.10 PREPARATION OF (INFECTED) CELL LYSATES FOR ANALYSIS OF PROTEINS

One 35mm dish per putative recombinant virus, plus two dishes for control conditions (one for a "mock" infection, using medium alone, and one

for infection with "wildtype" virus) were seeded with 1 x 10^6 cells each one day before use.

The medium was removed and each dish was incubated for one hour at room temperature with 200µl of the appropriate inoculum. The inoculum was replaced with 1.5ml TC100/5% FCS, and incubated under humidified conditions at 28°C, for the required time (for first analysis, usually 72 hours).

The cells were scraped into the medium using a large pipette tip, and transferred to a 1.5ml microcentrifuge tube. The cells were pelleted at low speed for one minute. The pellet was washed in PBS, and re-pelleted. All traces of PBS were removed, and the pellet resuspended in 50µl TE buffer. An equal volume of 2X PAG dye mix (10% SDS, 25% 2-mercaptoethanol, 50mM Tris-HCl, pH 6.8, 25% glycerol, a trace of bromophenol blue) was added, and boiled for 5 minutes. Samples not analysed immediately were stored at -20°C.

The lysates were then analysed by SDS-PAGE, and Western blotting, as described in sections 2.2.3.2 and 2.2.3.3.

After the initial analysis of recombinant virus proteins, all glucose transporter recombinant viruses were solubilised in the dye-buffer described in section 2.2.3.2.

2.2.2.11 PREPARATION OF HIGH TITRE VIRUS STOCKS

On the positive identification of a true recombinant virus a large stock was made for use in all further experiments using that virus.

A 200ml Spinner culture was prepared (as described in section 2.2.2.2) and grown until the cell density reached 5 x 10^5/ml. The cells were inoculated at a low MOI (multiplicity of infection) (0.1 to 0.2 pfu/cell), using the least passaged virus stock available that would achieve this (as estimated by titrating plaque assay, section 2.2.2.9). Infection was carried out by adding the required amount of inoculum to the culture, and returning to stir at 28°C. Infected cultures were incubated for 5 to 6 days (samples microscopically

analysed and counted using a haemocytometer daily, for determination of infection state). Supernatants containing the virus were harvested by centrifugation of the culture for five minutes at 10,000 rpm, and the removal of the supernatant from the cells. Supernatants were stored at 4°C. Two 1ml aliquots of each important virus passage were stored at -70°C.

2.2.3 PROTEIN ANALYSIS

2.2.3.1 CELL MEMBRANE PREPARATION

Cells in 35mm dishes were seeded at a density of $1x10^6$ cells/dish, one day prior to infection. The medium was removed, and the cells incubated with the appropriate inoculum for one hour. The medium was replaced, and the infected cells were incubated at 28°C for the required time (usually 48 or 72 hours).

The cells were scraped from the dishes, and pelleted gently in a microcentrifuge tube. The pellets were resuspended in PBS, and repelleted. This pellet was resuspended in 250µl ice cold TES buffer (10mM Tris-HCl; 5mM EDTA; 250mM sucrose; pH 7.2). Samples were stored on ice during the remaining procedure. Samples were homogenised, using 20 strokes of an electric Teflon homogeniser, transferred to a 3ml ultracentrifuge tube, and the volumes of each sample made up to 3ml by dilution and washing with ice cold TES buffer. Samples were spun at 100,000 g at 4°C, for 20 minutes, using a Beckman bench top micro-ultracentrifuge, and resuspended in 100µl TES buffer.

The 100,000 g_{max} fraction was taken as being the total membrane material of the cells. Where stated in the text, centrifugation at 16,000 g_{max} was used. This was taken as being a plasma membrane fraction.

Membrane samples for labelling studies were preferably used immediately, or stored at -70°C overnight. Samples for use in SDS-PAGE and

Western blotting only were stored in electrophoresis sample buffer (section 2.2.3.2) at -20°C, until required.

2.2.3.2 SODIUM DODECYL SULPHATE POLYACRLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoresis in the presence of SDS was performed using a method based on that of Laemmli (1970). Gels of 10, 12, or 15% polyacrylamide were used, made up using 0.248M Tris-HCl (pH 8.7) buffer, and polymerised by the addition of ammonium persulphate and N,N,N',N'-tetramethylethylene diamine (TEMED). The slab gel was poured, and overlaid with water-saturated butanol, until the gel had set. The butanol was then removed, and the top of the gel rinsed with distilled water. A 4% polyacrylamide stacking gel was made, using 0.345% Tris HCl (pH 6.8) buffer, and polymerised using ammonium persulphate and TEMED, as for the resolving gel, and a comb inserted to form the sample wells.

The protein samples were mixed with 6M urea sample buffer (10% SDS, 6M urea, 0.05% bromophenol blue), and incubated at room temperature for 20 minutes prior to loading.

Standard molecular weight markers were made up according to manufacturers instructions (Pharmacia LKB), and boiled in sample buffer (12.5% v/v 0.5M Tris-HCl (pH 6.8); 20% v/v glycerol, 2% w/v SDS; 5% v/v β-mercaptoethanol; and 0.00025% bromophenol blue) for five minutes before loading. Prestained markers were used when the gel was to be subjected to Western blotting. These were heated to 40°C for 10 minutes before loading, according to manufacturers' instructions.

The gel was run at a constant current overnight (the current depending on the percentage polyacrylamide in the gel, and whether one or two gels were being run on the apparatus at the time. Usually 20 to 25 mA/gel).

Following electrophoresis, initial protein analysis gels would usually be stained with Coomassie blue stain to determine whether the recombinant virus-expressed protein could be visualised in this way. Gels were stained for one hour at room temperature in Coomassie blue stain (0.25% Coomassie blue in 30% methanol, 10% acetic acid, 60% ddH_2O) and then destained in several changes of 30% methanol, 10% acetic acid, 60% ddH_2O, also at room temperature, until the background was adequately destained.

2.2.3.3 WESTERN BLOTTING OF PROTEINS FOLLOWING SDS-PAGE

A 3mm thick 10 or 12% SDS polyacrylamide gel was electrophoresed as described (section 2.2.3.2), removed from the glass plates, and trimmed using a razor blade.

3mm filter paper was cut to the size of the gel (12 pieces), as was one nitrocellulose sheet. Filter paper and nitrocellulose were pre-soaked in Western blotting buffer (48mM Tris-HCl; 39mM glycine; 0.375% w/v SDS, 20% v/v pure grade methanol; pH 9.2).

The filter paper, nitrocellulose membrane and gel were carefully assembled onto a semi-dry western blotting apparatus. The cathode was attached, and the transfer carried out at a constant 0.85mA/cm^2 (gel size), for an hour.

Protein transfer was briefly assessed by staining the membranes with Ponseau S stain (0.1% w/v Ponseau S; 3%w/v TCA) (if this was still unclear, the gel was stained with Coomassie stain, to assess the remaining protein). The Ponseau S was washed off with PBS-T buffer (PBS with 0.05% Tween-20 $\{C_{12}E_9\}$); and the membrane incubated at room temperature, with gentle shaking, in blocking buffer (PBS-T with 5% casein), for one hour.

The membrane was washed briefly in PBS-T, then incubated with the detecting antibody, with shaking, for at least an hour at room temperature. The antibody was diluted to the required amount, in PBS-T with 1% casein. The

membrane was rinsed briefly (PBS-T), followed by three ten minute PBS-T washes, with agitation. Incubation with the HRP (horse radish peroxidase)-labelled second antibody, diluted 1:2000 in PBS-T, was at room temperature, for at least an hour, with gentle shaking. The membrane was rinsed briefly, and then subjected to three ten minute washes, as before.

Immunoreactive proteins were detected using an ECL (enhanced chemiluminescence) Western blotting detection system, following the manufacturer's protocol. Processed membranes were subjected to autoradiography, using "hyperfilm" X-ray film, for 15 seconds in the first instance, with the period being adjusted according to the appearance of the first developed autoradiograph.

2.2.3.4 QUANTITATIVE IMMUNOBLOTTING

When some degree of quantitation was required from a Western blot, iodinated (^{125}I) protein A was used as the detection method, and parts of the procedure varied from those used in the ECL- detection Western blots.

The SDS-PAGE and transfer methods were as described (section 2.2.3.3). The blocking incubation was carried out at 4°C overnight in PBS-T with 3% casein. Whilst the washing procedures and the first antibody dilutions remained the same , the blot was incubated with the first antibody for at least two hours at room temperature. ^{125}I protein A [0.1μCi/ml] in PBS-T, 1% casein replaced the second antibody, and the blot was incubated with this at room temperature for two hours, with gentle agitation. The washing following this step involved six washes of five minutes each, with PBS-T. The blot was then air dried, and wrapped in cling film. The blot was then subjected to autoradiography at -80°C, using intensification screens; overnight in the first instance, and adjusted according to this result to optimise the clarity of the result.

On attaining a suitable autoradiograph from the blot, this was used as a template, to determine the relative positions of the transferred detected protein species. The appropriate, identified areas of nitrocellulose were the cut out and the radioactivity of each section was measured using a gamma counter. Areas of the blot away from any immunoreactive product (for example, within a sample lane but lower down than the immunoreactive product detected in that lane), of an equivalent size to the sample pieces, were taken and counted. These "background" control figures were subtracted from the experimental figures.

A series of known concentration standards (protein- depleted red blood cell membranes, prepared according to a method described by Gorga and Lienhard, 1981) were electrophoresed on the same gel as the experimental samples, and calculations of labelled protein concentration of each detected species were carried out directly on the basis of the measured standards' radioactive content. "Background" samples were taken for the RBC standard samples, and these figures subtracted before calculations were made. Calculations were made on the basis of the glut 1 content of red blood cell membranes prepared in this way being 600 pmol/mg protein.

2.2.3.5 TIME COURSE OF PROTEIN EXPRESSION

Cells, seeded at $1x10^6$ cells/35mm dish one day in advance, were infected at a known constant MOI with the recombinant baculovirus under examination. Two dishes per time point were allowed. Dishes of both mock infected cells (medium only "inoculum"), and "wildtype" (AcNPV:LacZ) virus of known MOI were also prepared.

Two dishes of recombinant virus-infected cells and one dish each of the control condition cells, were taken at each time point. (A visual microscopic analysis of the condition of the cells was made at this point). One dish of each condition, per time point was prepared as a cell lysate (see section 2.2.2.10,

using 6M urea sample buffer). The remaining dish of cells was used to prepare membranes for analysis (see section 2.2.3.1). Each set of prepared samples was stored at -20°C until all time course samples had been collected.

Sets of samples were examined and taken at zero, six , 12, 18, 24, 36, 42, 48, 60, 72, 96, and 120 hours post infection (hpi) .

Small samples of were retained for protein concentration estimation, before electrophoresis sample buffer was added.

Collected, prepared samples were subjected to SDS-PAGE. The control samples were stained using Coomassie blue. Recombinant virus infected samples were subsequently subjected to Western blotting using an appropriate antibody.

Samples were also subjected to quantitative immunoblotting (section 2.2.3.4), in order to determine the optimum expression of recombinant protein.

2.2.3.6 IMMUNOPRECIPITATION OF SAMPLES

Protein A:sepharose (7mg per sample- a sample corresponding to one 35mm dish of cells, or membranes from two such dishes) was swollen in cold 5mM phosphate buffer, pH 7.2, by gentle mixing (by rotation) at 4°C for 15 minutes. The protein A:sepharose was loosely pelleted at low speed in a microcentrifuge, and washed twice with cold phosphate buffer (with repelleting).

Anti-glut1 antiserum, 80µl/sample, was added to the washed Protein A :sepharose, and resuspended in phosphate buffer to a total microcentrifuge tube volume of 1.3ml. The mixture was allowed to coagulate by gentle mixing at 4°C for two hours.

The mixture was loosely pelleted and washed twice as before. An equal volume of resuspended mixture was added to each prepared sample (in microcentrifuge tubes), and gently mixed at 4°C for two hours, to immunoprecipitate the sample.

The immunoprecipitate was gently pelleted (at low speed, in a microcentrifuge) and washed with 1ml wash solution (0.2% thesit detergent (nonaethylene glycol dodecyl ether; $C_{12}E_9$), with mixed proteinase inhibitors; (antipain, aprotinin, pepstatin A and leupeptin, each at 1μg/ml), in ice cold 5mM phosphate buffer, pH7.2). This was done six times (if an observable reduction of pellet volume was noted, this was reduced to four washes).

The final pellet was resuspended in 150μl 6M urea sample buffer (10% SDS, 6M urea, 0.05% bromophenol blue), and gently mixed at 4°C for ten minutes. The sepharose was pelleted by microcentrifugation before the sample was loaded on an SDS-PAG for electrophoresis.

Immunoprecipitated samples were subjected to SDS-PAGE, as described in section 2.2.3.2; but using a 3mm thick gel, and a broad-tooth comb to ensure sufficient size to accommodate the whole sample.

2.2.3.7 CYTOCHALASIN B LABELLING

Cytochalasin B labelling experiments were carried out on either membrane preparations of infected cells (as prepared by the protocol in section 2.2.3.1), or on whole cells. Cells, in 35mm culture dishes, were infected with the appropriate virus(es) for the required period. The medium was removed, and centrifuged at low speed, so that detached cells could be washed in PBS, re-centrifuged, and returned to the dish, after the monolayer had been washed twice with PBS buffer. The detached cells were returned to the dish in a minimal volume of PBS.

1.6μCi/ml ^{3}H-Cytochalasin B (sp. act. 19.6 Ci/mmol); 10^{-4}M Cytochalasin E, and 100mM D-glucose -where appropriate, were added to a microcentrifuge tube, in a total volume of 150μl (made up with PBS). The photosensitive cytochalasin B was added last, in the dark. The mixture was gently pipetted onto the monolayer, and irradiated with UV light for 45 seconds, at room temperature. 1ml solubilisation buffer (2% thesit detergent,

with mixed protease inhibitors (as used in section 2.2.3.6), in 5mM phosphate buffer pH 7.2) was added to each dish. The cell / solubilisation buffer mixture was transferred to a microcentrifuge tube and mixed, by gentle pipetting to solubilise the cell matter. The tubes were incubated for 20 minutes, then microcentrifuged at high speed at 4°C for 20 minutes. The supernatant was removed to a fresh tube, and immunoprecipitated (see section 2.2.3.6).

The samples were electrophoresed on 3mm thick 10 or 12% SDS-polyacrylamide gels.

The gels were lightly stained using Coomassie blue stain, and destained such that the positions of the sample lanes could be determined, and that the positions of the molecular weight standard protein markers could be seen.

Each sample lane was carefully isolated, using a razor blade, and accurately cut into 0.5mm slices (using carefully spaced razor blades set in a perspex block). Each slice was transferred to a scintillation vial, and incubated in an oven at 80°C until the slice was dry and hard. The positioning of the molecular weight standards was noted relative to the slicing of the experimental lanes. The dried sample slices were dissolved by a two hour incubation at 80° C, in 0.5ml of hydrogen peroxide, 5% ammonia. The vials were allowed to cool, and scintillation fluid was added, and the radioactivity of each sample was counted using a scintillation counter. The results were analysed graphically, relative to the molecular weight standards.

2.2.3.8 ATB-BMPA LABELLING

As for Cytochalasin B labelling, ATB-BMPA labelling was carried out on either membrane samples, or whole cells.

The cells were washed twice in PBS (or, if membranes, membrane samples from two 35mm dishes were resuspended in PBS). The cells were incubated with 100μCi ATB-[-2-^{3}H]-BMPA photolabel in a total volume of

300µl PBS, in darkness, at 18°C. The cells were then irradiated (in 35mm dishes) for 45 seconds in a Rayonett photochemical reactor. 1ml of Thesit detergent buffer per sample (2% $C_{12}E_9$ in 5 mM sodium phosphate pH 7.4, with mixed proteinase inhibitors as described in section 2.2.3.6) was used to solubilise the cells or membranes. From this point onwards the procedure used is as section 2.2.3.7. following cell solubilisation.

2.2.3.9 PROTEIN ESTIMATION ASSAY

The BCA protein estimation assay was used. BSA standard protein concentrations of 0, 2, 4, 6, 8, and 10µg were prepared by diluting 2mg/ml stock BSA to 1mg/ml using 0.2M NaOH. The standards were each made to 10 µl volume using 0.1M NaOH.

5µl of sample protein was made up to 10µl with 0.2M NaOH.

10ml of reagent A (1% BCA-NO_2 detection reagent, 2% $Na_2CO_3.H_2O$ [11.5g (4.6%) $NaCO_3$. 10 H_2O], 0.16% Sodium tartrate, 0.4% Sodium hydroxide, and 0.95% $NaHCO_3$: Made upto pH 11.25 using 1M NaOH, and filtered through a 0.7µm filter (Millipore).) was mixed with 200µl of reagent B (4% $CuSO_4$, filtered through a 0.7µm filter (Millipore)) to make reagent C.

200µl reagent C was added to each sample, including the standards, and incubated at 37°C for 30 minutes.

The absorbence was determined spectrophotometrically, using a microtitre plate reader, at 650nm. Sample protein concentrations were estimated using the standard protein samples. All samples were analysed in duplicate, and means of readings used in the estimations.

2.2.3.10 HEXOSE TRANSPORT ASSAYS

Cells were infected with virus (at high MOI, inoculated for one hour at room temperature) for the required period, in 75cm^2 flasks. 0.3µCi ^{3}H-2-

deoxy-D-glucose was made up to a 100μM concentration with unlabelled 2-deoxy-D-glucose. If inhibitor (fructose, glucose, or both) was added, this was to a concentration of 0.3M.

Infected cells were PBS washed twice, and the cells of each condition were counted, in order to account for differences in cell death and growth etc. Samples were also retained for protein concentration estimation.

Transport was followed at 28°C for the required periods. Zero time, two minutes, and five minutes were the usual times, per condition. The assay was stopped by the addition of 3ml stop solution (10mg/ml phloretin in PBS). The cells were pelleted at 20 000rpm for two minutes at 4°C. The supernatant was removed, and the cell pellets resuspended in a further 3ml stop solution. The cells were re-pelleted and solubilized in 500μl 10% TCA. The soluble material was transferred to a scintillation vial, scintillant was added, and samples were counted using a Packard 1500 scintillation counter.

CHAPTER 3.

CONSTRUCTION AND PRODUCTION OF RECOMBINANT VIRUSES

A rabbit GLUT1 cDNA (Asano et al, 1988) was used as source DNA for all the experiments described in this thesis. Once produced and identified, the constructs were co-transfected into the Sf9 insect cells together with linearised baculovirus (AcMNPV) DNA for the production of recombinant baculoviruses, which were then identified by DNA and protein analysis.

3.1.1 PRODUCTION OF WHOLE GLUT1 BACULOVIRUS CONSTRUCT

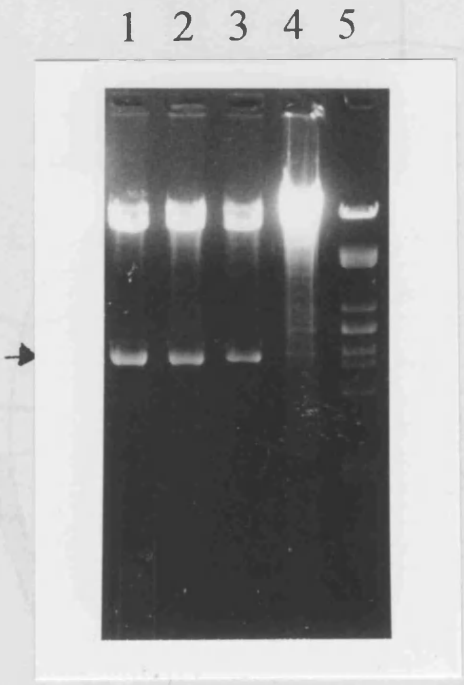
A 2610 base pair fragment was isolated from pUC19:GLUT1, using Eco RI sites in the 5' and 3' noncoding regions. This corresponded to base pairs -123 to 2487 (where base pairs 1 to 1600 represent the GLUT1 coding region), and incorporated 123 5' non-coding base pairs, and 888 base pairs of the 3' non-coding region.

This was gel purified (by Gene Cleaning), and ligated into Eco RI digested (non-dephosphorylated) pVL 1392 baculovirus transfer vector. This was then used to transform competent *E.coli*. A number of colonies were picked and small scale plasmid preparations were made. Each of these was digested with Eco RI, and three were found to contain a fragment of an appropriate size, when visualised by gel electrophoresis (see figure 3.1). Further restriction analyses were carried out to confirm the identity of the band, and to determine it's orientation in the vector. Figure 3.2 represents a partial restriction map of the pVL:GLUT1 construct, and figure 3.3 outlines the restriction analysis.

Since all of the three clones were positively identified as GLUT1 DNA, and found to be correctly orientated, two were chosen to be amplified as large scale plasmid preparations.

3.1.2 PRODUCTION OF RECOMBINANT BACULOVIRUS E4.2

The transfer construct pVL:GLUT1 was co-transfected into insect cells with linearised AcNPV DNA, and the resulting virus progeny was screened for



Lane 1	Eco RI digested pVL1391:GLUT1 clone 9
Lane 2	Eco RI digested pVL1391:GLUT1 clone 5
Lane 3	Eco RI digested pVL1391:GLUT1 clone 4
Lane 4	Eco RI digested pVL1392
Lane 5	pst 1 digested λ DNA

Figure 3.1 Agarose gel electrophoresis of Eco RI digested pVL1392:GLUT1 clones 4, 5 and 6, identified from twelve minipreps (photograph not available) as positives. Identification of Eco RI fragments of ~2610 base pairs, corresponding to the glut1 Eco RI fragment.

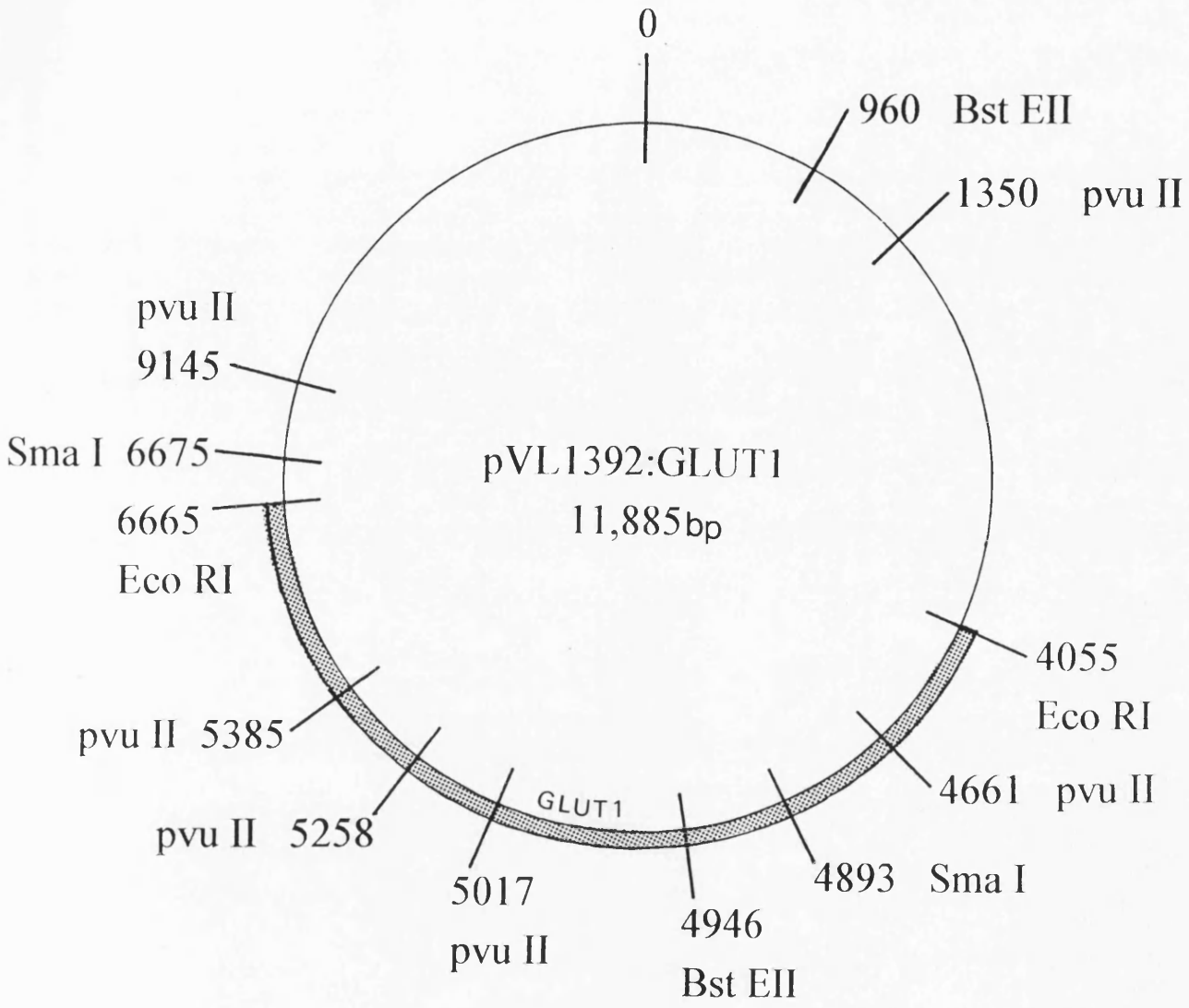


Figure 3.2 Construction of vector pVL1392:GLUT1 from pVL1392, containing the GLUT1 Eco RI fragment from pUC19:GLUT1, inserted in the correct orientation. Limited restriction enzyme recognition sites, used for analysis and orientation determination, are shown (not drawn to scale).

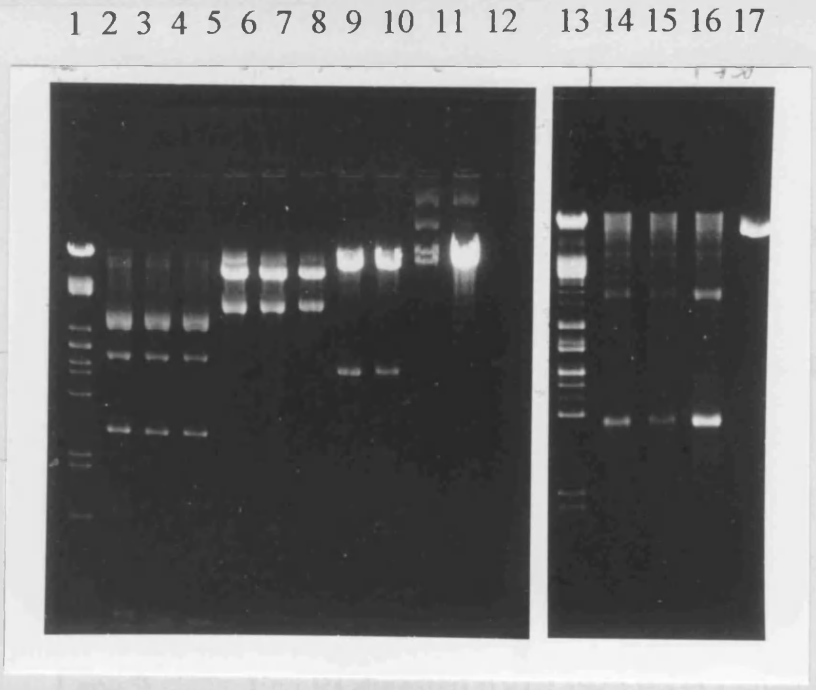


Figure 3.3 Agarose gel electrophoresis of restriction digestion of pVL1392:GLUT1 clones 4, 5 and 9, identified as putative positives, with restriction enzymes Eco RI, Bst EII, pvu II and pst I. Single enzyme digestions to determine the orientation of the GLUT1 Eco RI fragment in each of the clones. See figure 3.2 for partial restriction maps. Listings of the digests performed are over the page. The restriction profiles of each of the three clones show that all correspond to the correct orientation.

Digests shown in figure 3.3

Lane 1	pst I digested λ DNA.
Lane 2	pvu II digested pVL1392:GLUT1 clone 9
Lane 3	pvu II digested pVL1392:GLUT1 clone 5
Lane 4	pvu II digested pVL1392:GLUT1 clone 4
Lane 5	Bst EII digested pVL1392:GLUT1 clone 9
Lane 6	Bst EII digested pVL1392:GLUT1 clone 5
Lane 7	Bst EII digested pVL1392:GLUT1 clone 4
Lane 8	Eco RI digested pVL1392:GLUT1 clone 9
Lane 9	Eco RI digested pVL1392:GLUT1 clone 5
Lane 10	Eco RI digested pVL1392:GLUT1 clone 4
Lane 11	Eco RI digested pVL1392
Lane 12	Eco RI fragment of GLUT1 from pUC19:GLUT1
Lane 13	pst I digested λ DNA
Lane 14	pst I digested pVL1392:GLUT1 clone 9
Lane 15	pst I digested pVL1392:GLUT1 clone 5
Lane 16	pst I digested pVL1392:GLUT1 clone 4
Lane 17	pst I digested pVL1392

recombinants by plaque assay. Eight putative recombinants were picked, and subjected to a round of plaque purification (see section 2.3.6) to ensure there was no contamination of the recombinants with wildtype virus. Small passage 2 stocks were made of four of the putative recombinant viruses, which were then subjected to a second round of plaque purification. Cells were then infected with these putative recombinants, and cell lysates made at 72 hpi (hours post infection), which were subjected to SDS-PAGE followed by Western blotting using the anti-C-terminal anti-peptide antibody. Figure 3.4 is a Western blot analysis of two putative recombinants. Clearly, virus E4.2 is expressing a protein of around 50kDa, which is highly immunoreactive with the anti-GLUT1 antibody. The virus was subjected to a third round of purification plaque assay, and passaged again, until a titre of at least 1 x 10^7 pfu/ml had been achieved. Large stocks were made in 250ml Spinner culture. Virus titres were determined by plaque assay, for each passage, and are summarised in table 4.1 below. The final passage of E4.2 ,p3s ("s" denoting spinner culture derivation) which was used in all subsequent experiments was found to have a titre of between 8.5 x 10^6 pfu/ml and 2.6 x 10^7 pfu/ml (estimates from two separate assays)

Virus passage number (monolayer or spinner culture derived, m or s.)	titre (pfu/ml)
0m	2.00 x 10^3
2m	2.05 x 10^6
3m	6.50 x 10^6
3s (first est.)	2.60 x 10^7
3s (second est.)	8.50 x 10^6

Table 4.1 Recombinant baculovirus E4.2 titres

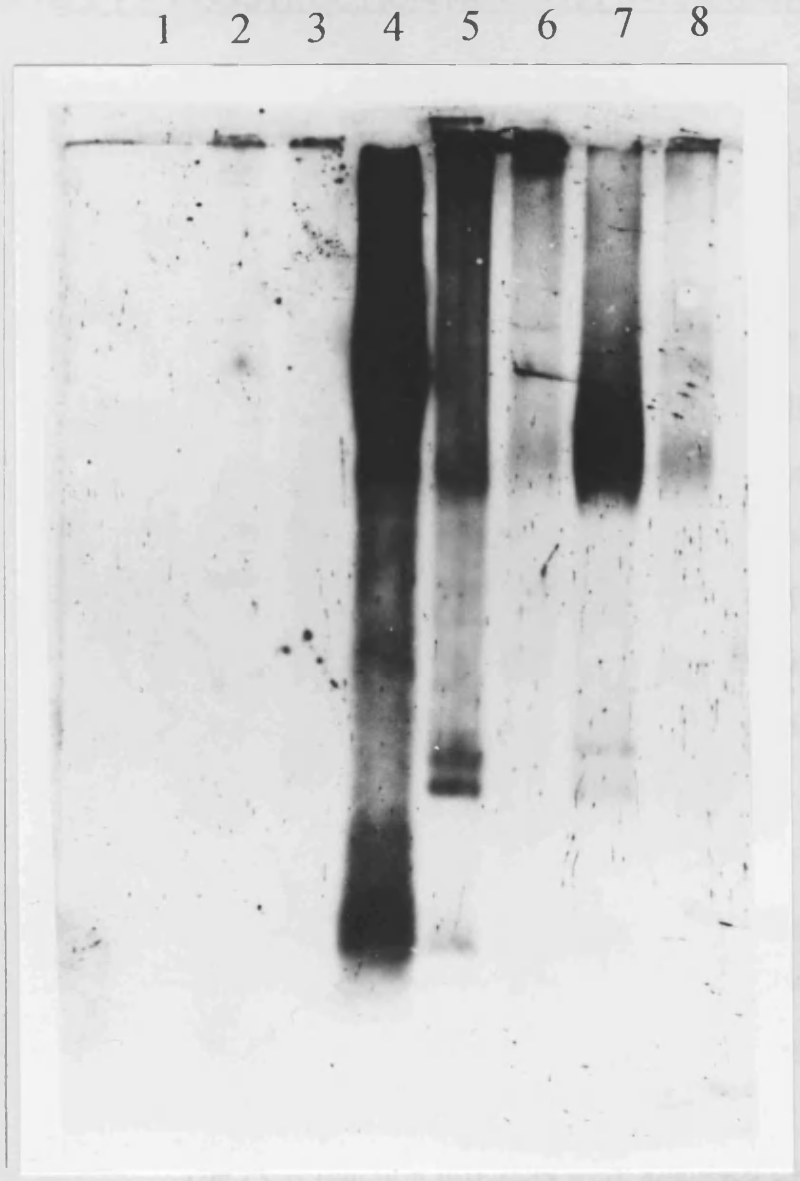


Figure 3.4 Western blot analysis of Sf9 cell lysates at 72 hpi. Lane 1; prestained markers; Lane 2 mock infected Sf9 cells; Lane 3, AcMNPV:LacZ infected cells; Lane 4 erythrocyte membranes; Lane 5 cells infected with putative recombinant virus E4.3; Lane 6 cells infected with putative recombinant E4.7; Lane 7 cells infected with putative recombinant E4.2. The blot was detected using the anti C-terminal anti-GLUT1 antibody. Note that virus E4.2 has a strong immunoreactive band at around 50 kDa, in contrast to the strong immunoreactive band of ~ 55 to 65 of the erythrocytes.

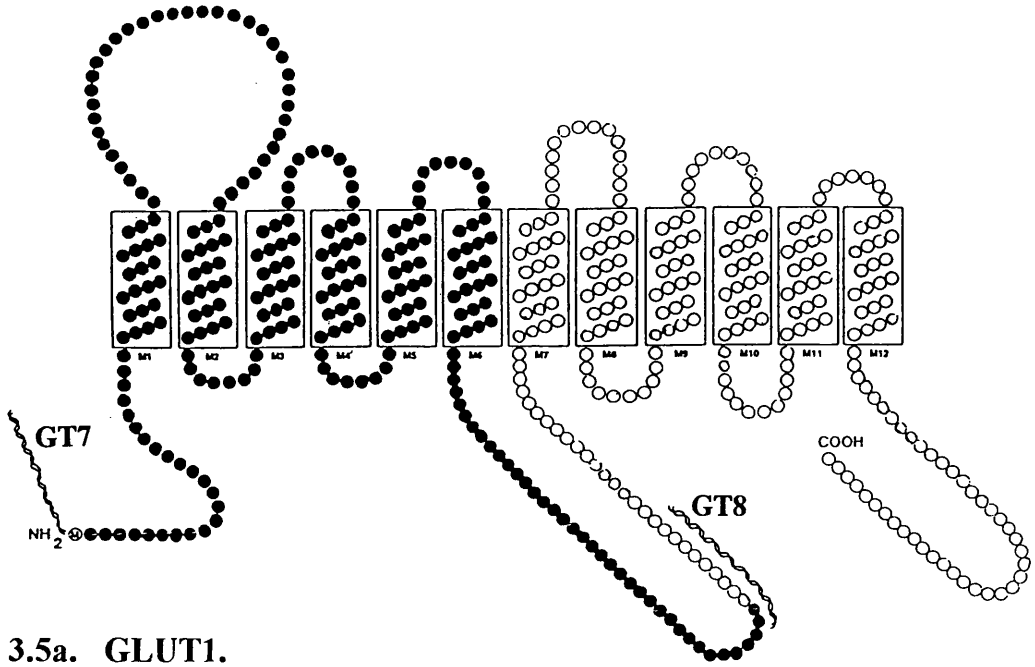
3.2.1 CONSTRUCTION OF GLUT1 N-TERMINAL BACULOVIRUS.

The N-terminal "half" of the GLUT1 gene was isolated and amplified by PCR. Figure 3.5a represents the approximate positioning of the oligonucleotides used in the PCR reaction, in relation to the two-dimensional protein structure.

Oligonucleotide GT7 was made to the last 25 5' non-coding base pairs of the GLUT1 gene, modified to incorporate an Eco RI restriction enzyme recognition site (see figure 3.5b). The gene's own start codon was retained in the PCR product, and was not included in the oligonucleotide.

A complementary strand oligonucleotide, GT8 was designed to an area of the cDNA from base pairs 907 to 939; corresponding to part of the large cytoplasmic loop between transmembrane domains six and seven, as indicated on the protein structure representation in figure 3.5a. This construct was designed so that there would be an overlap of this construct with the C-terminal "half" construct of 11 amino acids at the central loop. This oligonucleotide was designed to incorporate a Bgl II site, and two stop codons (replacing those coding for Phe 263 and Ser 265), such that the last amino acid encoded is Ala 267. Figure 3.5c outlines the design of GT8.

The PCR reaction products were analysed by agarose gel electrophoresis. A clear band corresponding to an 810 bp product was found, corresponding to the expected size of GT78 (see figure 3.6). This was gel purified, and cloned into the plasmid pT7blue, on blunt ends (see sections 2.2.10 and 2.2.11). Small scale plasmid preparations of the resulting clones were analysed by restriction digestion with Eco RI and Bgl II (still contained within the insert, since cloning into pT7blue retained the restriction sites encoded by the oligonucleotides), in order to identify the GT78 insert. Three clones contained inserts of the appropriate size. Samples of each were subjected to PCR using the original oligonucleotide primers, and the results



3.5a. GLUT1.

ACT GCC AGA ATT CCT AGC GCA GGG C

Eco R1 site

3' noncoding GLUT1

3' noncoding GLUT1 (GLUT1 original Met start codon immediately follows primer

3.5 b. Oligonucleotide GT7

GAT GGG CTA CAT CTA GGC AGG TCA GCG GAA GAG

stop stop

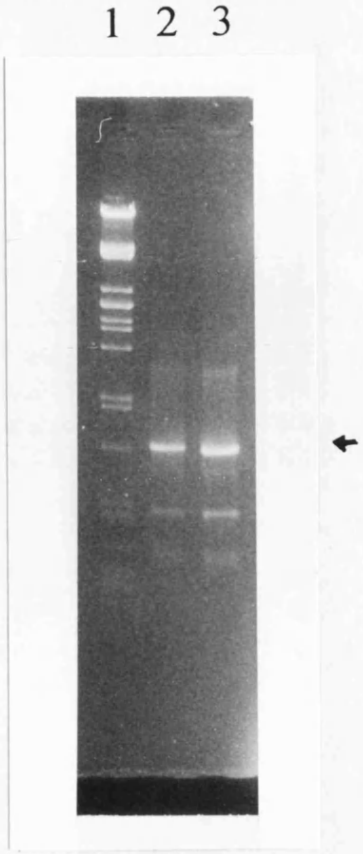
Bgl II site

Complementary sequence to GLUT1 coding bases 947 to 940

Complementary sequence to GLUT1 coding bases 926 to 907.

3.5c. Oligonucleotide GT8

Figure 3.5 **a.** Cartoon representation of the two-dimensional structure of the GLUT1 protein, showing representations of the relative positioning of the PCR oligonucleotides GT7 and GT8. **b.** Sequence and design of oligonucleotide GT7. **c.** Sequence and design of oligonucleotide GT8.



Lane 1 pst I digested λ DNA

Lanes 2 & 3 PCR products using GT7 and GT8

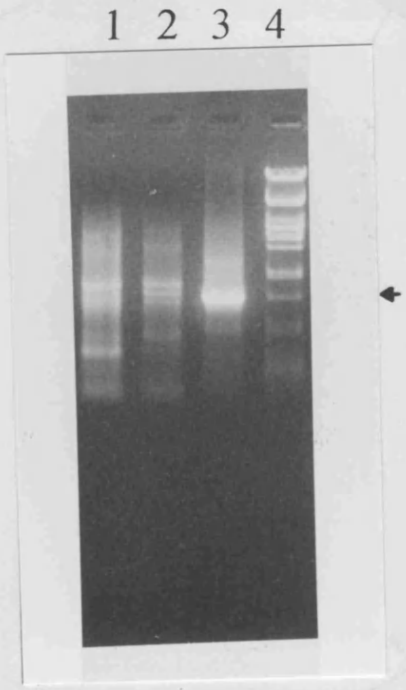
Figure 3.6 Agarose gel electrophoretic analysis of PCR products from GLUT1 cDNA template (pUC19:GLUT1) using oligonucleotides GT7 and GT8. PCR reaction involved 30 cycles as described in section 2.2.7.

analysed by agarose gel electrophoresis. Clone 9 was found to produce a clear product at the appropriate size (see figure 3.7). For further confirmation, this construct was subjected to restriction digestions. Figure 3.8 shows a limited restriction map of the construct, showing the enzymes used in the analysis. Figure 3.9 shows the agarose gel analysis of the digests, which positively identify clone 9 as containing the GT78 insert. A large scale plasmid preparation was carried out, and the clone designated pT7blue:GT78. The GT78 insert was removed by sequential overnight restriction digestion with Eco RI and Bgl II, and gel purified, including additional phenol:chloroform extraction and ethanol precipitation steps in the Gene Clean procedure, to ensure no vector contamination. Baculovirus transfer vector pVL1393 was also digested with these enzymes.

Of 96 colonies from the cloning procedure, 24 were picked, small scale preparations made, and subjected to digestion with the two restriction enzymes used in the cloning. Figure 3.10 shows the results of electrophoresis of 12 such digests, and illustrates that every clone contains an insert of the appropriate size. A single representative clone was selected for further analysis. Figure 3.11 outlines the restriction digests carried out, and the confirmation of the identification of GT78. Figure 3.12 shows limited restriction site information for the plasmid pVL1393:GT78. A large scale plasmid preparation was made, and designated pVL1393:GT78.

3.2.2 PRODUCTION OF RECOMBINANT BACULOVIRUS N1

Medium from the co-transfection of AcNPV DNA with pVL1393:GT78 transfer construct was screened by plaque assay. Six putative recombinant viruses were picked, and the remaining co-transfection medium retained. These were each subjected to a purification plaque assay, from which nine white plaques were taken, and subjected to another round of plaque assays to ensure their purity. These viruses were passaged to small stocks and three



Lane 1	PCR using pT7blue:GT78 clone 10
Lane 2	PCR using pT7blue:GT78 clone 8
Lane 3	PCR using pT7blue:GT78 clone 9
Lane 4	pst I digested λ DNA

Figure 3.7 Agarose gel electrophoresis of PCR products, using putative pT7blue:GT78 cloned DNA as templates (1μl of standard small scale DNA prep.), and using oligonucleotides GT7 and GT8 as primers (as described in section 2.2.7). Clone 9 is clearly identified as having a clear band corresponding to the ~810 base pairs of the GT78 PCR fragment.

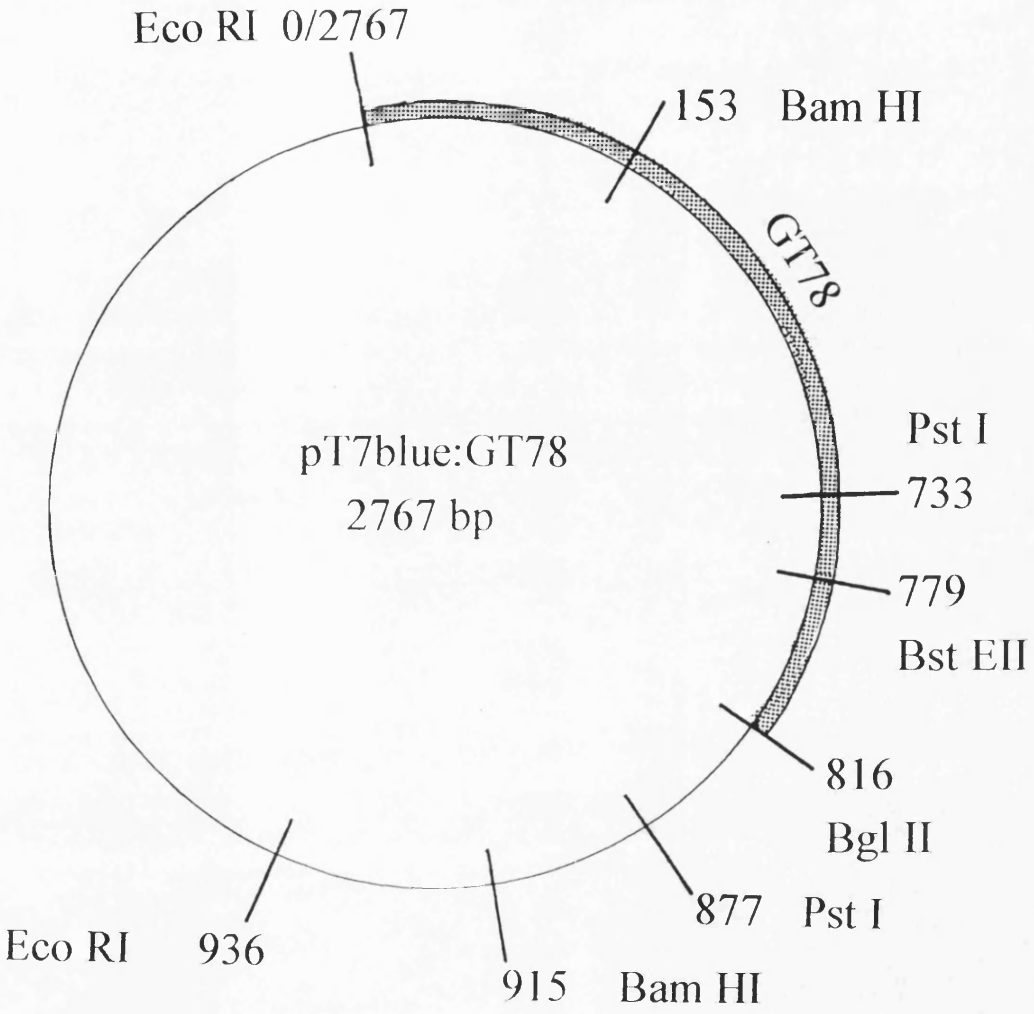
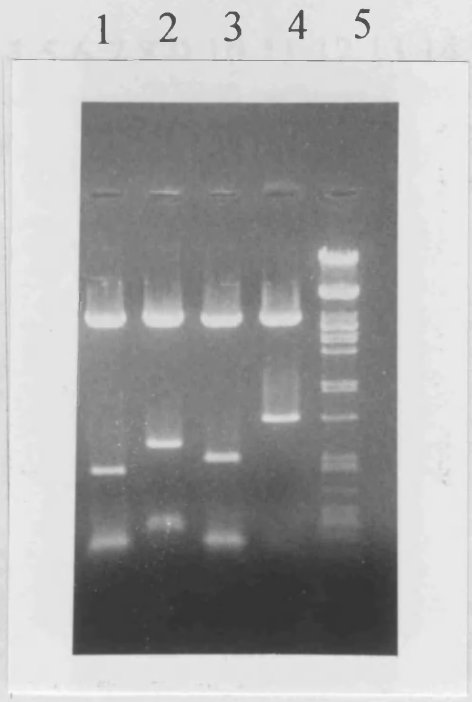


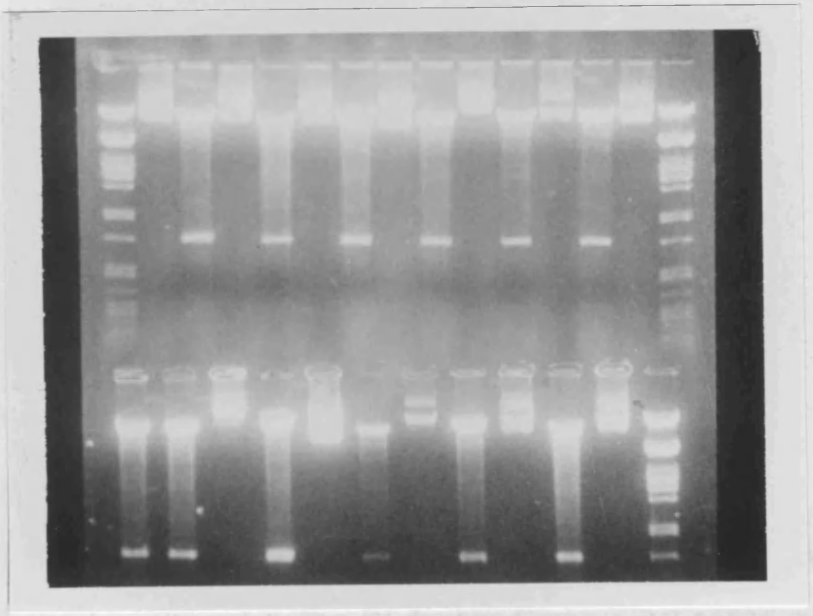
Figure 3.8 Schematic representation of construct pT7blue:GT78, from the vector pT7blue, and the PCR product GT78. Restriction recognition sites used in analyses of the constructs are shown (not drawn to scale).



Lane 1	Bam HI and pst I double digest of pT7blue:GT78 clone 9
Lane 2	Bam HI and BstEII double digest of pT7blue:GT78 clone9
Lane 3	Eco RI and pst I double digest of pT7blue:GT78 clone 9
Lane 4	Eco RI and Bgl II double digest of pT7blue:GT78 clone 9
Lane 5	pst I digested λ DNA

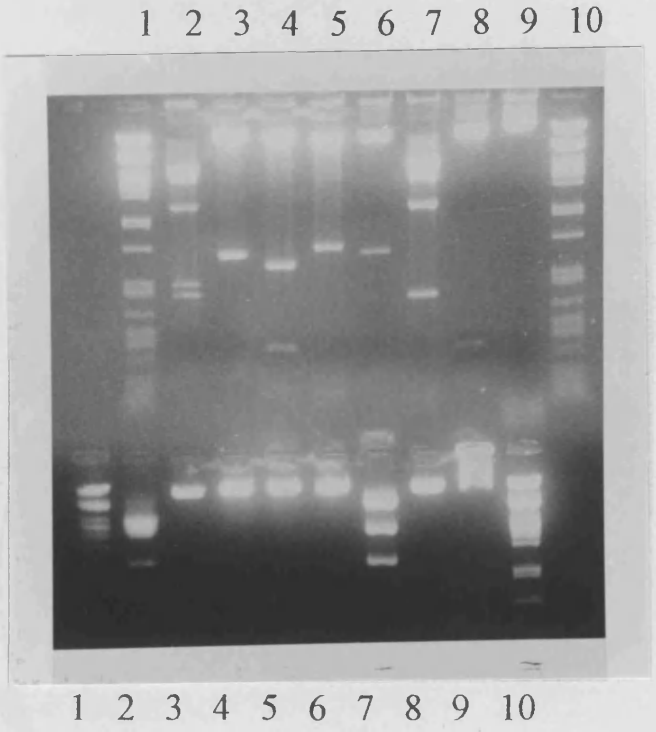
Figure 3.9 Restriction digest analysis of pT7blue:GT78 clone 9. Digestion with Eco RI and Bgl II, Eco RI and pst I, Bam HI and Bst EII, and Bam HI and pst I.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



16 17 18 19 20 21 22 23 24 25 26 27

Figure 3.10 Agarose gel electrophoresis of restriction digestion of putative pVL1393:GT78 clones, with restriction enzymes Eco RI and Bgl II (enzymes used in the directional cloning of GT78 into the vector pVL1393). Lanes 1, 15 and 27 represent λ DNA digested with pst I, as a molecular size marker. 12 clones have been analysed. On the top row, from lane 2, undigested then double digested DNA has been loaded alternately, for each clone. On the bottom row, double digested then undigested DNA for each clone has been loaded from lane 17. Lane 16 represents the double digestion of one clone (corresponding undigested sample not shown). Every clone analysed was found to contain a fragment of the appropriate size (~810 base pairs).



Lane 1 pst I digested λ DNA

Lane 2 Eco R1 & pvu II double digested pVL1393/ pVL1393:GT78

Lane 3 Sma I & Eco RI double digested pVL1393/ pVL1393:GT78

Lane 4 Bam HI & Bgl II double digested pVL1393/ pVL1393:GT78

Lane 5 Eco RI & Bgl II double digested pVL1393/ pVL1393:GT78

Lane 6 Sma I digested pVL1393/ pVL1393:GT78

Lane 7 pvu II digested pVL1393/ pVL1393:GT78

Lane 8 Bam HI digested pVL1393/ pVL1393:GT78

Lane 9 Undigested pVL1393 or pVL1393:GT78.

Lane 10 pst I digested λ DNA

Figure 3.11 Agarose gel electrophoresis of restriction digest analysis of a pVL1393:GT78 clone, using restriction enzymes Bam HI, pvu II, Sma I, Eco RI and Bgl II, Bam HI and Bgl II, Sma I and Eco RI, and Eco RI and pvu II. The lower part of the gel represents the digests carried out on pVL1393 alone; and the upper part of the gel represents the same digests carried out on a pVL1393:GT78 clone.

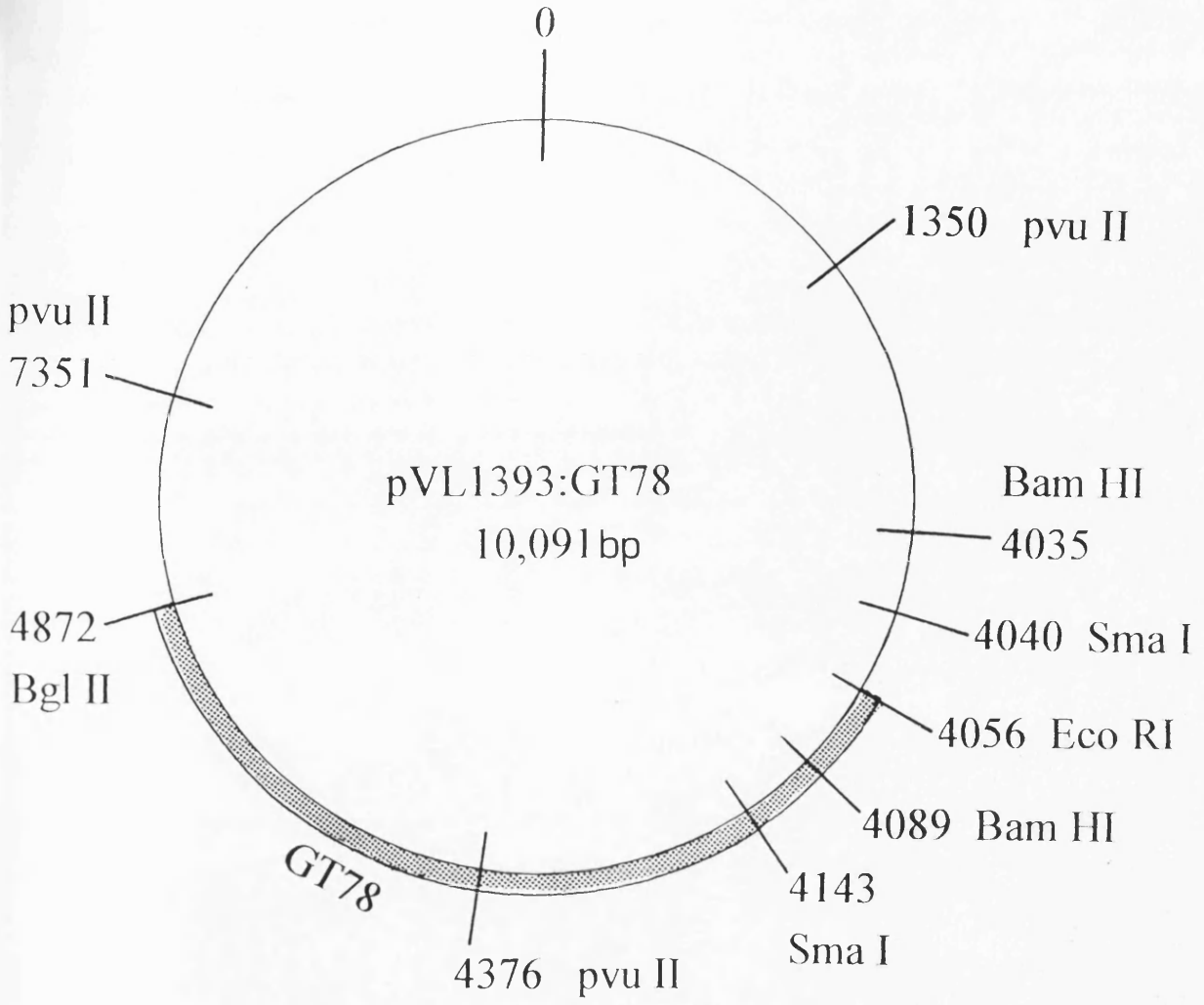


Figure 3.12 Schematic representation of construct pVL1393:GT78, from the vector pVL1393, and the GT78 Eco RI; Bgl 11 fragment from pT7blue:GT78. Restriction recognition sites used in analyses of the constructs are shown (not drawn to scale).

were used to infect cells for Western blot analysis at 72 hpi, using the anti N-terminal anti peptide antiserum (see section 2.2.3). Figure 3.13 shows that Sf9 cells infected with viruses N1 and N4.1 express an immunoreactive protein with an apparent molecular weight of 31kDa, which is within the predicted range for the protein from construct GT78. This protein is not present in mock infected cells, cells infected with the "wildtype" virus AcNPV:Lac Z, cells infected with virus E4.2, or cells infected with virus R12. None of the detected bands reacted with the anti C-terminal antibody.

Virus N1 was passaged to p2, and a large passage 3 (p3) stock was produced in Spinner culture (p3s). The titre of this was 1.2 x 10^7, and this stock was used for all subsequent experiments involving the expressed N-terminal half of GLUT1.

The table below summarises the titres for each passage of virus N1, monitored to gain a reasonable titre at as low a passage number as possible.

Virus passage number (monolayer or suspension culture derived)	titre (pfu/ml)
1m	1.05 x 10^6
2m	1.23 x 10^7
3s	1.20 x 10^7

Table 3.2 Recombinant baculovirus N1 Titres

3.3.1 PRODUCTION OF C-TERMINAL HALF GLUT1 BACULOVIRUS CONSTRUCT

The C-terminal half of GLUT1 was isolated by the PCR amplification of the corresponding area of the GLUT1 DNA. The PCR product was first

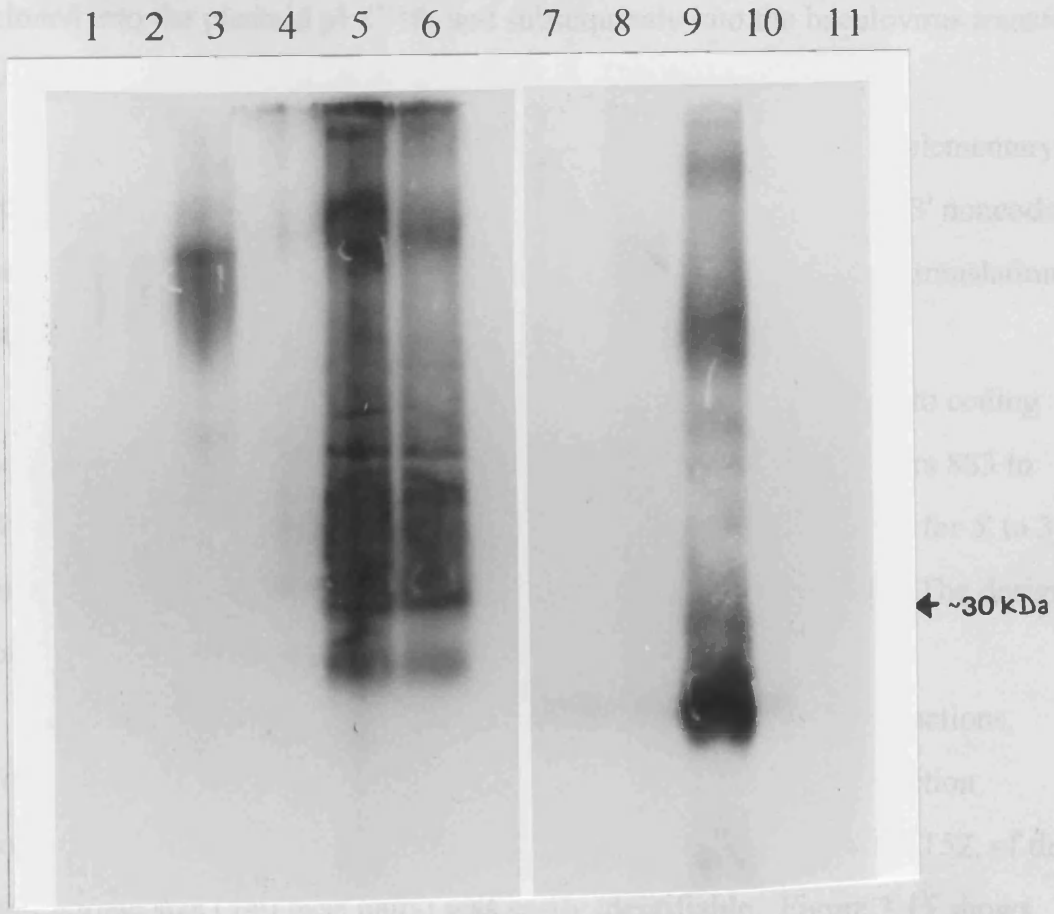


Figure 3.13 Western blot analysis of Sf9 cells; mock infected (lane 1); infected with AcMNPV:LacZ virus (lane 2); recombinant baculovirus E4.2 (full-length GLUT1) (lane 3); recombinant baculovirus R12 (C-terminal half)(lane 4); putative recombinant baculovirus N1 (lane 5); putative recombinant baculovirus N4.1 (lane 6). Lanes 1 to 6 detected using the anti-N-terminal half anti-GLUT1 antibody. Lanes 7 to 11 represent the same samples as in lanes 2 to 6 (in the same order), but the anti-C-terminal GLUT1 antibody was used in the detection. All cell lysates were made at 72 hpi. Both putative recombinant baculoviruses N1 and N4.1 are shown to have an immunoreactive band of around 30 kDa M_r ,when using the anti-N-terminal half GLUT1 antibody, which is appropriate to the size of the N-terminal "half " of GLUT1 as designed. No immunoreactive product is detectable from infection with either of these viruses, when detecting with the anti-C-terminal anti-GLUT1 antibody.

cloned into the plasmid pUC 18, and subsequently into the baculovirus transfer vector pVL941.

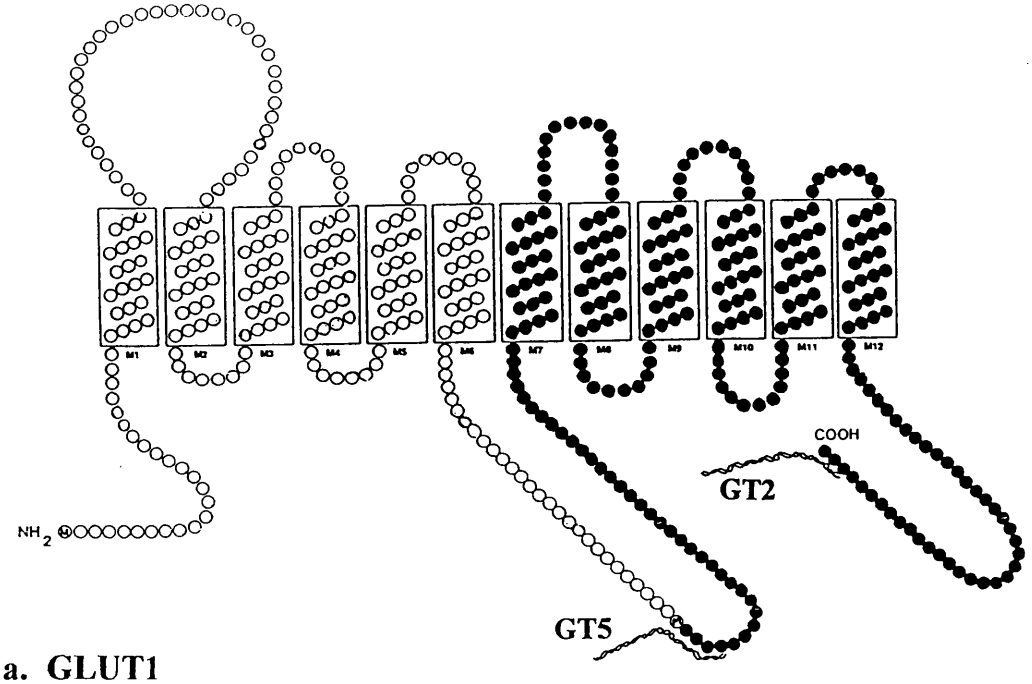
A 30 base oligonucleotide, GT2B was designed to be complementary to the 3' end of the GLUT1 coding sequence, as well as part of the 3' noncoding sequence, incorporating an Xba I restriction recognition site, and a translation stop codon (see figure 3.14b).

GT5 is an oligonucleotide of 27 base pairs, corresponding to coding sequence in the central loop region of the GLUT1 protein (base pairs 883 to 897), including a Bam HI site and translation start codon.(designed for 5' to 3' amplification during PCR, as indicated by arrows in figure 3.14a). The design of GT5 is outlined in figure 3.14c.

The oligonucleotides GT2B and GT5 were used in PCR reactions, following conditions outlined in section 2.2.7. 10% of the final reaction mixture was analysed by agarose gel electrophoresis. A product, GT52, of the appropriate size (740 base pairs) was easily identifiable. Figure 3.15 shows GT52 against a fragment the size of the entire GLUT1 coding region (GT21). The 740bp band was excised, and purified by phenol, phenol:chloroform and chloroform extractions, and an overnight ethanol precipitation.

GT52 was digested overnight with Bam HI and cloned into pUC18 on the Bam HI cloning site. A clone containing an appropriately sized fragment was identified by small scale plasmid preparation from recombinant colonies, and restriction digestion with Bam HI. This clone was amplified by large scale plasmid preparation. A sample of this DNA was digested with Bam HI for four hours and subjected to agarose gel electrophoresis. The GT52 fragment was gel purified (using the gene clean method), and further purified by ethanol precipitation.

This was finally cloned into pVL941 (a baculovirus transfer vector) on the Bam HI cloning site. Two clones containing a 740 base pair fragment were isolated and amplified as large scale DNA preparations. These clones ,



a. GLUT1

CTC GGA TCC ATG GAG AAG AAG GTC ACC

Bam HI site

Translation start codon

Sequence corresponding to GLUT1 coding bases 883 to 987

b. Oligonucleotide GT5

GAG GCA GTG TCT AGA GCC TCA CAC TTG GGA

Sequence complementary to 3' noncoding GLUT1

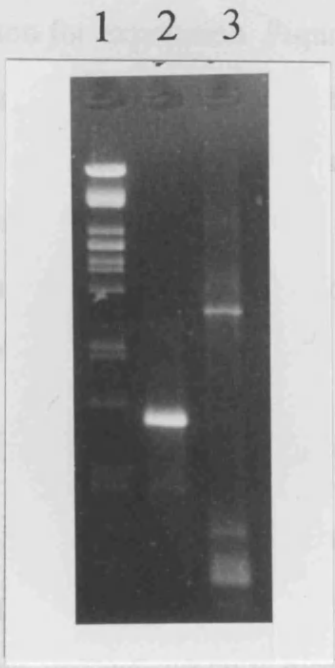
Xba I site

Translation stop codon

complementary to end of coding sequence GLUT1

c. Oligonucleotide GT2

Figure 3.14 **a.** Simplified schematic representation of the proposed two-dimensional structure of the GLUT1 protein, showing the approximate relative positions of the oligonucleotides GT5 and GT2. **b.** Sequence and design of oligonucleotide GT5. **c.** Sequence and design of oligonucleotide GT2.



Lane 1 pst1 digested λ DNA

Lane 2 GT52

Lane 3 GT21

Figure 3.15 Agarose gel electrophoresis of the PCR product from pUC19:GLUT1, using oligonucleotides GT2 and GT5 (PCR conditions given in section 2.2.7), shown against a PCR product representing the full-length coding region of glut 1 (1470 bp), GT21, and λ DNA digested with pst I, as a standard marker. A single band of ~740 bp is clearly visible.

pVL941:GT52, 9 and 10, were subjected to restriction analysis, based on restriction sites known to exist in the C-terminal half of the original GLUT1 cDNA. This analysis served as confirmation of the identity of the clones, as well as identification of whether the fragment was inserted into the vector in the correct orientation for expression. Figure 3.17 gives details of the digests carried out and figure 3.16 shows limited restriction maps of pVL 941 alone, and pVL941:GT52 with the GT52 insert correctly orientated for expression.

The digests confirmed the identity of both inserts as GT52, and showed pVL941:GT52 clone 9 to be in the correct orientation, and clone 10 to be incorrectly orientated.

3.3.2 PRODUCTION OF RECOMBINANT BACULOVIRUS R12

Following a similar procedure to those described in sections 3.1.2 and 3.2.2, a number of putative AcNPV:GT52 recombinants were picked. Following an arduous process which is discussed in section 3.4, virus R12 was finally isolated as expressing protein of ~25 kDa that was highly immunoreactive with the anti C-terminal anti-peptide antibody. Figure 3.18 illustrates protein species immunoreactive with this protein from mock infected Sf9 cells, erythrocytes, and Sf9 cells infected with a number of putative GLUT1 C-terminal half-expressing recombinant baculoviruses.

3m	6.00×10^5
4m	1.50×10^6
5m	1.45×10^6
5s	1.25×10^7

Table 3.3 Recombinant baculovirus R12 titres

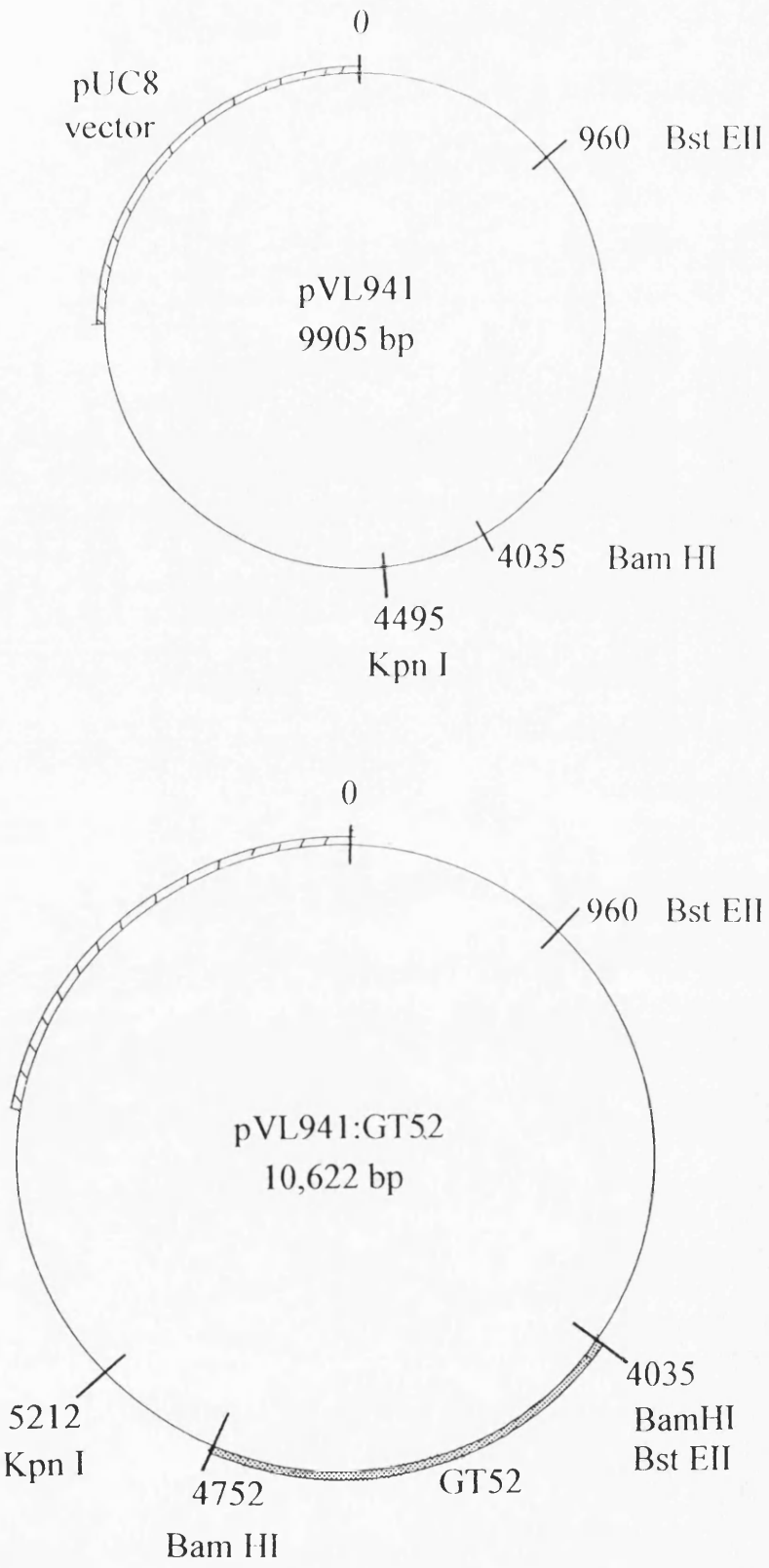
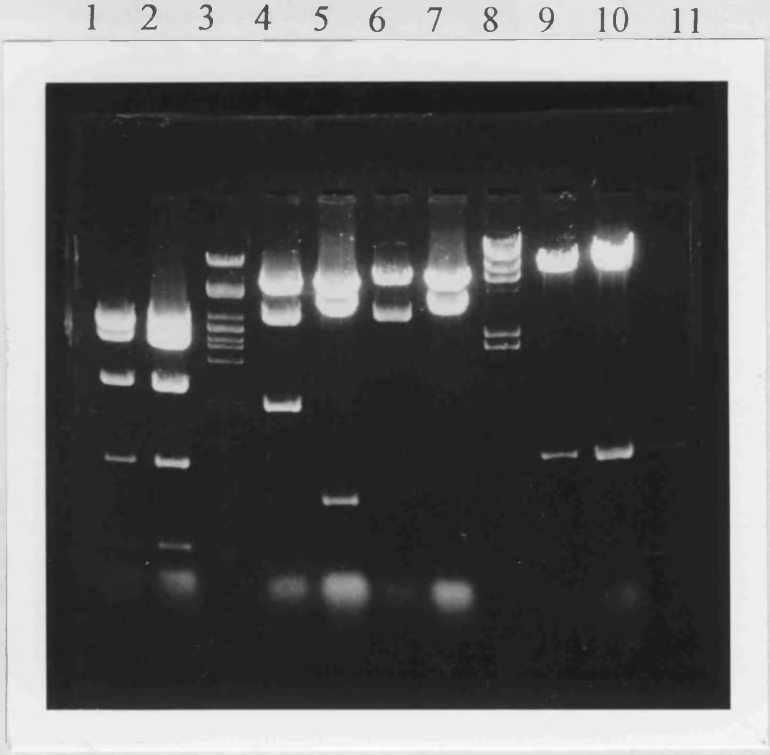


Figure 3.16 Schematic representations, showing limited restriction enzyme recognition positions of the baculovirus transfer vector pVL941 (top); and the construct pVL941:GT52 (shown with the GT52 insert correctly orientated).



Lane 1	pvu II digested pVL941:GT52 clone 10
Lane 2	pvu II digested pVL941:GT52 clone 9
Lane 3	pst I digested λ DNA
Lane 4	Bst EII & Kpn I double digested pVL941:GT52 clone 10
Lane 5	Bst EII & Kpn I double digested pVL941:GT52 clone 9
Lane 6	Bst EII digested pVL941:GT52 clone 10
Lane 7	Bst EII digested pVL941:GT52 clone 9
Lane 8	Hind III digested λ DNA
Lane 9	Bam HI digested pVL941:GT52 clone 10
Lane 10	Bam HI digested pVL941:GT52 clone 9
Lane 11	Bam HI GT52 fragment excised from pUC18:GT52.

Figure 3.17 Agarose gel electrophoresis of restriction digests of pVL941:GT52 clones 9 and 10. The digests carried out are indicated above. The restriction digestion profiles of both clones positively identify them as pVL941:GT52. Digestion with pvu II, Bst EII, and Bst EII and Kpn I double digest show that only clone 9 is correctly orientated in the vector.

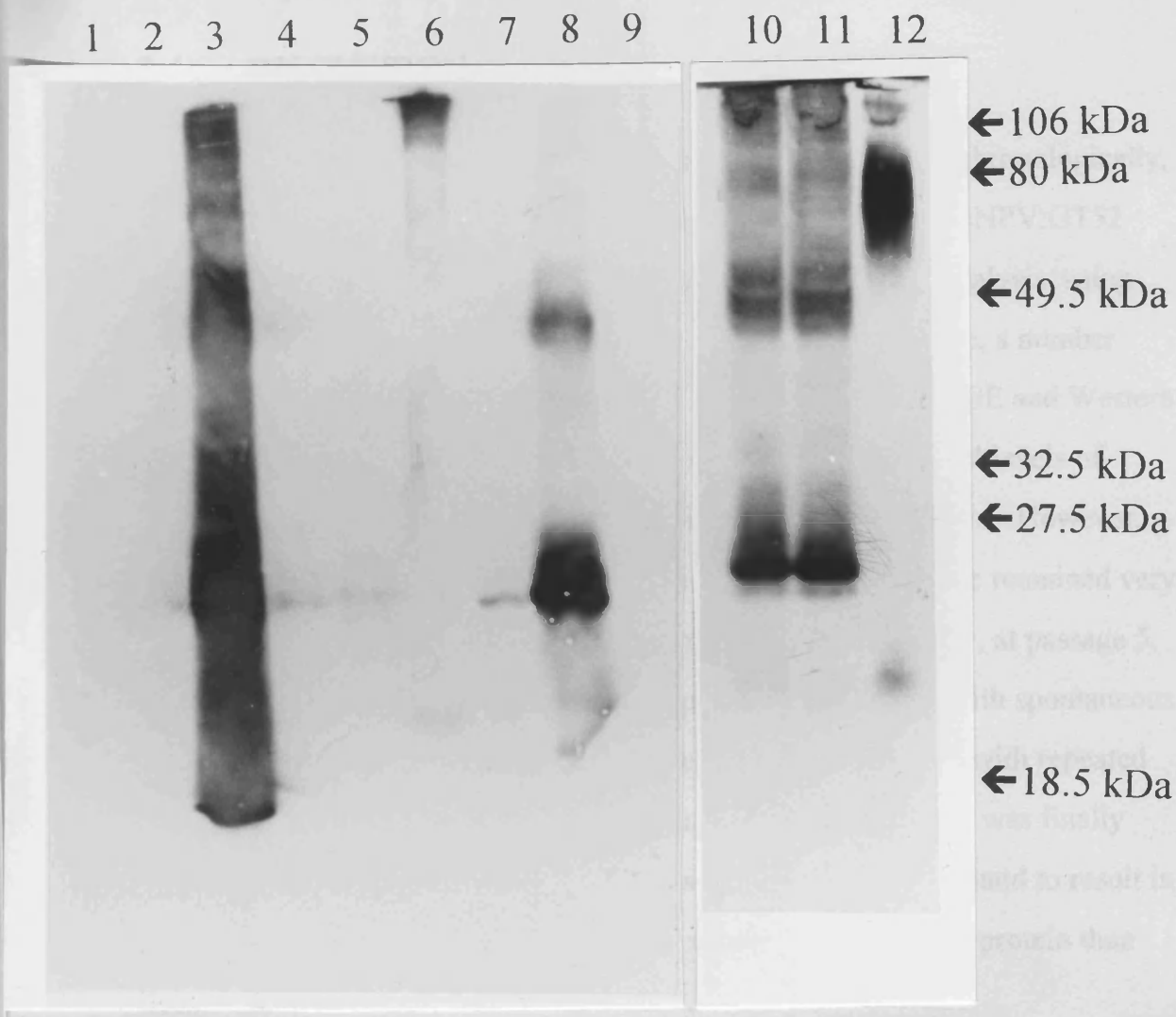


Figure 3.18 Western blot using the anti C-terminal anti-GLUT1 antibody. Sf9 cells were infected with either TC100 only (mock infection) (lane 1); AcMNPV:LacZ (lane 2); recombinant baculovirus R12 (lane 3); putative recombinant baculovirus R8 (lane 4); putative recombinant baculovirus R4 (lane 5); or recombinant baculovirus R13 (lane 8)(each at passage 5); and lysates made at 72 hpi. Erythrocyte membranes (unclear) were run in lane 6. Other putative recombinant viruses were run in lanes 5 and 9. Viruses R12 and E13 clearly show immunoreactive protein species at around 25 kDa. Lanes 10 and 11 more clearly demonstrate the immunoreactive profile of R12 infected cells, shown against RBC (lane 12)(separate Western blot). Equal quantities of total protein were loaded in all of the Sf9 cell lanes (for each gel). Molecular weight standards correspond to the blot showing lanes 10 to 12.

3.4 DISCUSSION

The AcMNPV:GT52 construct was the first construct, chronologically, to be co-transfected into Sf9 cells. Initially, thirteen putative AcNPV:GT52 recombinants were picked, and screened by immuno-dot-blot analysis, using the anti C-terminal anti peptide antiserum as detection. Of these, a number were used to infect cells which were then subjected to SDS-PAGE and Western blotting, as described. Virus R13 was chosen as producing good levels of expression of a 25 kDa GLUT1 antibody immunoreactive protein. However, despite passaging the virus in culture a number of times, the titre remained very low. The best titre attained (from the best estimate) was 3 x 10^6, at passage 5. Since the passage number is rather high (problems associated with spontaneous mutation and other genetic reorganisations are often associated with repeated passaging of viruses in culture), and the titre still low, this virus was finally abandoned in favour of another. R12 was screened later, and found to result in apparently improved levels of expression of the C-terminal half protein than R13.

Table 3.3 illustrates the problems of low titre that were experienced for virus R13. The titres measured for R12 were an improvement on this, and passaging to passage 5 (Spinner) produced a sufficiently high titre for further work, at 1.25 x 10^7.

Virus	Passage number (monolayer or spinner derived)	Estimated titre (pfu/ml)
R13	2s	245
R13	3m	3.00×10^2
R13	5m (1^{st} est.)	3.00×10^6
R13	5m (2^{nd} est.)	1.00×10^4
R12	3m	6.00×10^5
R12	4m	1.50×10^6
R12	5m	1.45×10^6
R12	5s	1.25×10^7

Table 3.4 Comparison of titres of viruses R13 and R12

CHAPTER4.

OPTIMISATION OF EXPRESSION AND QUANTITATION OF PROTEIN PRODUCTS

4.1 FULL LENGTH GLUT1 PROTEIN

Figure 3.4 (chapter 3) illustrates the positive identification of anti GLUT1 antibody reactive protein expressed by cells infected with the recombinant baculovirus E4.2. It is clear that the whole GLUT1 protein being expressed in this way shows a greater mobility than the GLUT1 protein expressed natively in human erythrocyte membranes. That is, where the native GLUT1 is seen as a smeary band with an M_r of about 55 to 60 kDa, the E4.2 protein, whilst also smeary, appears at a M_r of about 50kDa.

4.1.1 QUANTITATION AND LOCALISATION OF GLUT1 PROTEIN EXPRESSION BY E4.2

In order to determine the level of GLUT1 expression being achieved in Sf9 cells by this virus, quantitative immunoblotting of an SDS-PAGE gel was carried out. This is shown in figure 4.1; and the calculated quantities of expression of whole GLUT1 protein by virus E4.2 are summarised below. Calculations were made on the basis of standard quantities of RBC of known concentration being processed with the experimental samples, based on 600pmol GLUT1/mg RBC membrane protein.

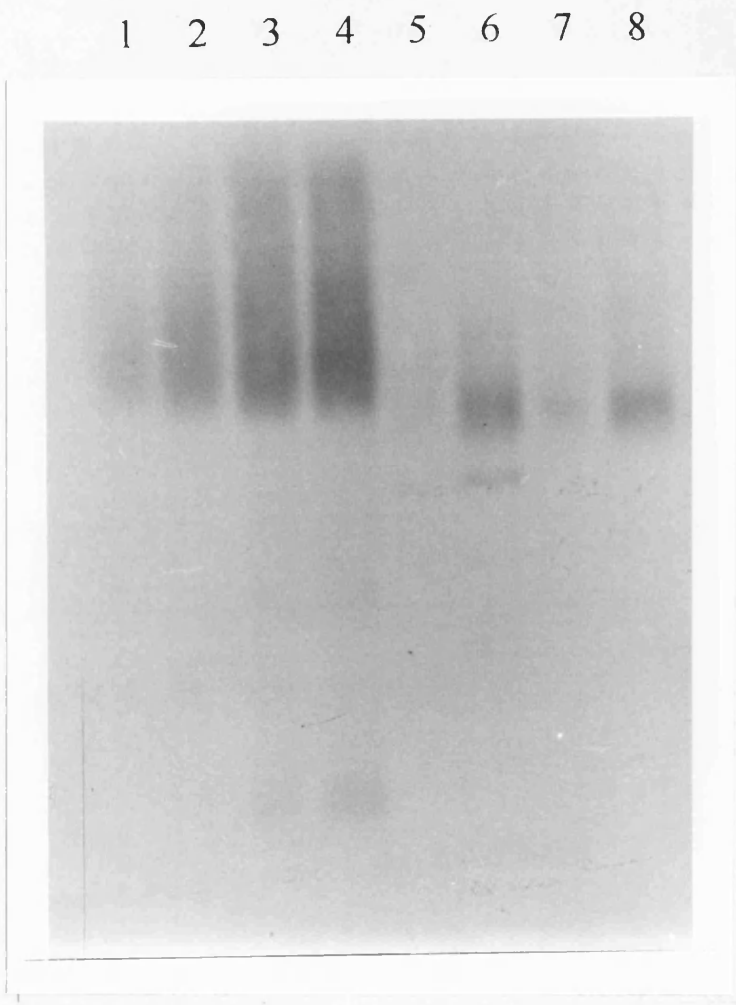


Figure 4.1 Western blotting of a 12% SDS-PAGE of Sf9 cell lysates (lanes 5 and 7), and membrane preparations (lanes 6 and 8) of cells infected with E4.2, taken at 48 hpi. (lanes 5 and 6 contain 5μg total protein, lanes 7 and 8, 20μg) Lanes 1 to 4 represent varying quantities of RBC membranes (est. 5, 10, 20 and 30μg respectively). The blot was processed according to section 2.2.3.4.

SAMPLE	RBC membrane immunoreactive equivalent (μg)	pmol glut1/mg protein
5μg membranes	1.6	192
20μg membranes	3.4	102
mean membrane		147
5μg whole cell lysates	1.7	204
20μg whole cell lysates	3.3	99
mean cell lysate		151.5

Table 4.1 Quantification of E4.2 GLUT1 protein

These figures indicate that the level of expression of GLUT1 protein in Sf9 cells infected with recombinant baculovirus E4.2 is approximately a quarter of the GLUT1 expressed natively in erythrocyte membranes. However, a number of factors must be considered. Firstly, although background radioactivity was accounted for in the calculations, the physical size of the immunoreactive band is not fully taken into account in this procedure. That is, the molecular weight range over which the principal immunoreactive species is found in erythrocytes is significantly greater than the range over which the E4.2/Sf9 expressed GLUT1 protein is found. Therefore, the radioactivity of the immunoreactive species is measured over a wider area in the red blood cell lane of the blot. Even if equivalent quantities of immunoreactive protein are present in each sample, physical constraints of the nitrocellulose for the immobilisation of protein may create a maximal band intensity per unit area, and therefore protein that is more "spread out" may be more accurately measurable.

The figures shown in table 4.1 suggest that a maximal loading of the nitrocellulose may be occurring, and that perhaps only lower band intensities should be used in the calculation of quantitation. That is, the figures calculated from an initial loading of 5mg of total protein (for both whole cell lysates, and membrane preparations) are very noticeably higher (at 192 pmol/mg and 204 pmol/mg for membranes and whole cells) than those from a loading of 20mg total protein (102 and 99 pmols/ml for membrane samples and whole cell lysates respectively).

From this then, a mean of around 150 pmol GLUT1 molecules per mg total Sf9 membrane protein is calculated. However, considering the possibility of overloading the nitrocellulose with one immunoreactive species, this figure may actually be closer to 200 pmol/mg.

These quantification experiments were carried out more than a year after the production of the passage 3s stock of virus E4.2. Some reduction in virus titre is to be expected on storage of baculovirus stocks. However, on the visual comparison of Western blotting of E4.2 samples when the stocks were new, and after a year or so of storage, suggests a very significant reduction of the titre of this virus. For example, figure 6.1 shows similar band intensities for E4.2 and the R12 monomer band. Figure 6.1 also apparently shows that the E4.2 GLUT1 band is of an approximately equivalent intensity (though without the same molecular weight distribution) to the erythrocyte equivalent band. Therefore, the level of expression of GLUT1 protein by Sf9 cells using the recombinant baculovirus E4.2 was apparently higher than the calculated 150 pmol/mg, earlier in the project. In an even later quantitation experiment, the level of GLUT1 protein from E4.2 infected insect cells was calculated to be only 25 pmol/mg. This is a certain indication of deterioration of the virus.

Although there is no recognised reliable method currently available for the isolation of the cytoplasmic membrane fraction of insect cells, differential centrifugation to approximately separate cytoplasmic membranes from total

cellular membrane, and analysis of total membrane fraction compared to whole cell lysates were considered to be the extent of this determination.

SDS-PAGE and Western blotting was carried out on equal protein quantities of membrane and whole cell samples from cells infected with E4.2. Western blots of whole cell lysate samples of the E4.2 infected cells, show the E4.2 protein product (50kDa) to be either undetectable, or only detectable on prolonged exposure of the immunofluorescent blot to film. However, membrane preparations made of cells identically infected, very clearly and consistently show the E4.2 protein band. This can be seen in figure 4.2. This seems to be clear indication of membrane localisation of this protein.

Differential centrifugation (2.2.3.1) in order to determine the percentage of the GLUT1 protein that is expressed at the cell surface was carried out. Although the blots were very poor and unclear, calculations indicated that between 70% and 115% of the E4.2 protein was present in the 16,000 g_{max} centrifugation. This, with the previous data, apparently confirms that a majority of the E4.2-expressed GLUT1 protein is localised to the cytoplasmic membrane.

4.1.2 DETERMINATION OF OPTIMAL PROTEIN EXPRESSION

The Western blots carried out for the initial identification of the desired proteins from cells infected with the recombinant viruses all used cell lysates made at 72 hpi, since it would be expected that if protein would be detectable at all, it would be seen by this point in the infection cycle. That is, each of the recombinant proteins are expressed under the control of the polyhedrin promoter, which generally dictates that relatively large quantities of protein would be present at this stage in an infection. However, since recombinant protein expression is part of a viral infection which eventually kills the cells involved, a full time course of expression was undertaken.

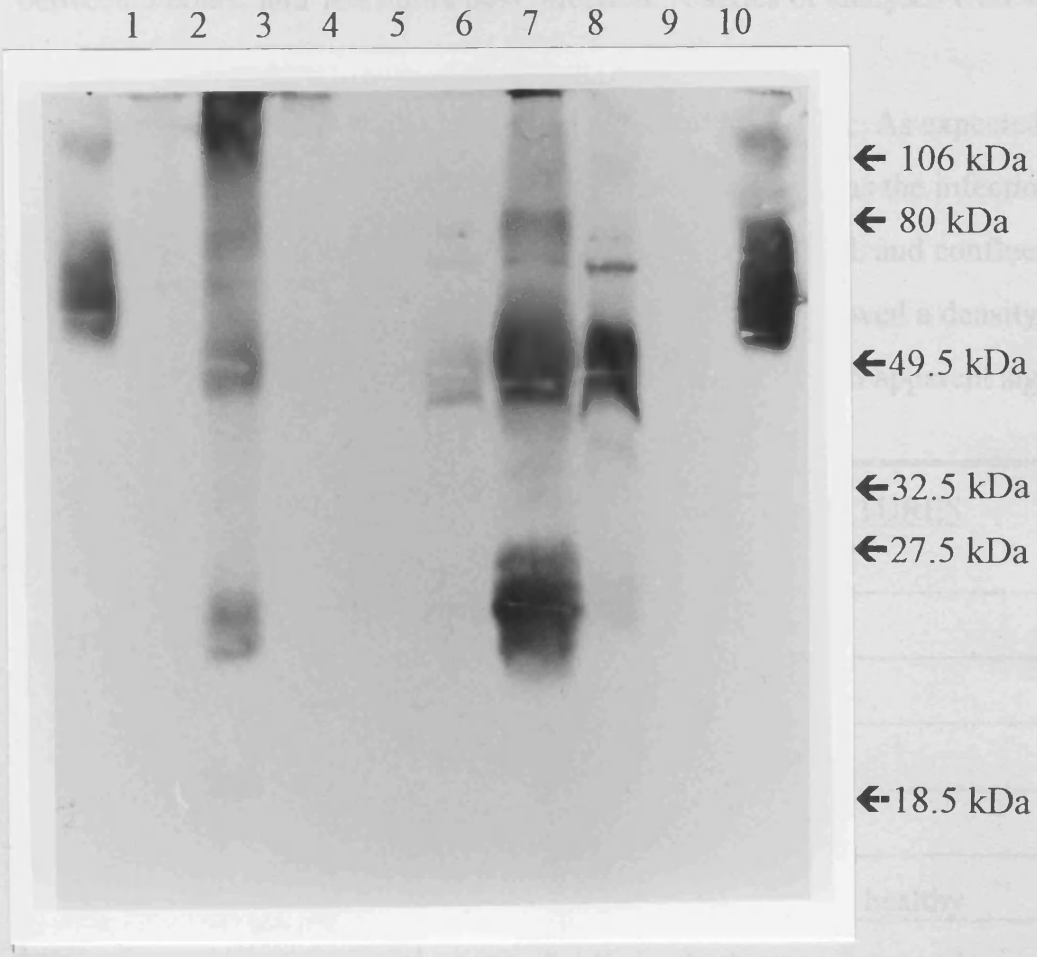


Figure 4.2 Western blotting of Sf9 cell lysates (lanes 2,3,4 and 5) and membrane preparations (6,7,8 and 9) of cells infected with AcMNPV:LacZ; the recombinant baculovirus R12; recombinant baculovirus E4.2, and mock infected Sf9 cells respectively. For E4.2, cells were infected with 1.13 pfu/cell virus, and all lysates or membranes were made at 48 hpi. Small samples were taken for protein estimation, and 20μg protien per sample was loaded onto the 12% SDS-PAG. RBC membranes were run in lanes 1 and 10. The Western blot was carried out using the anti-C-terminal peptide antibody.

A time course analysis, as described in section 2.4.5 was undertaken for the infection of Sf9 cells with the E4.2 recombinant virus. As summarised in table 4.2, samples of infected cells were taken at regular time points, between 0 hours, and 168 hours post infection. A series of analyses were made at each timepoint.

Visual analysis was made of cells at each time point. As expected, there was a gradual decline in the cells' healthy appearance, as the infections proceeded. At 36 hpi, the cell growth was obviously retarded, and confluence was at about 50% (the uninfected cells control condition showed a density of around 80%), though these cells themselves were showing no apparent signs of

TIME POST INFECTION (hours)	MAIN VISUAL FEATURES
0	Cells infected at time 0
6	
12	
24	
36	40 to 50% confluence. Appear healthy
48	~20% of cells beginning to appear sick
56	Significant evidence of cell death
60	>60% cells appear sick. ~10% dead (floating)
72	
96	
120	>50% cells floating (dead) <5% appear healthy
168	Most cells dead or dying. No evidence of healthy cells

Table 4.2 Visual analysis of time course of infection with E4.2

deterioration (crenellation of the outer membranes being the most visual indication of cell deterioration). By 48 hpi, an estimated 20% of the cells had begun to take on the characteristic appearance of infected cells. By 72 hpi more than 60% of the cells had this appearance, and an estimated 10% had become detached from the plastic surface, presumed dead. By 120 hpi, only very few cells (<5%) had a healthy appearance, and by 168 hpi, at least 50% of the cells were dead, with no evidence of healthy cells remaining.

Cells were harvested and lysates made, and immediately frozen at each point, for analysis on completion of the course. Figure 4.3 is the Western blot analysis of whole cell lysates over the time course. A standard volume, corresponding to a mean estimated total protein concentration of 20mg per sample was used (although protein concentrations varied per sample, see fig 4.4, using a standard volume gives an indication of the total amount of GLUT1 protein expressed in a culture dish, rather than relative to the total cell protein). This analysis shows that no GLUT1 protein is detectable before 24 hpi, and that from around 48 hpi onwards, no particularly noticeable differences in quantity can be visually detected.

Figure 4.5 is a Western blot showing membrane samples taken at each timepoint. Again, a constant volume was loaded per sample, corresponding to a mean of 20mg per sample (of membrane protein). It is clear, on comparing this to figure 4.3, that there is more immunoreactive material present in 20mg of membrane protein than is present in 20mg of whole cell protein, suggesting targeting of this protein to the membrane fractions of the cell. Again, no material is visually detectable before 24 hpi, but samples taken at 48, 54 and 60 hpi appear to contain more immunoreactive material than either earlier or later samples. From this, it appears that an optimum expression is occurring at around these times. Since visual analysis of the cells indicates that they are still

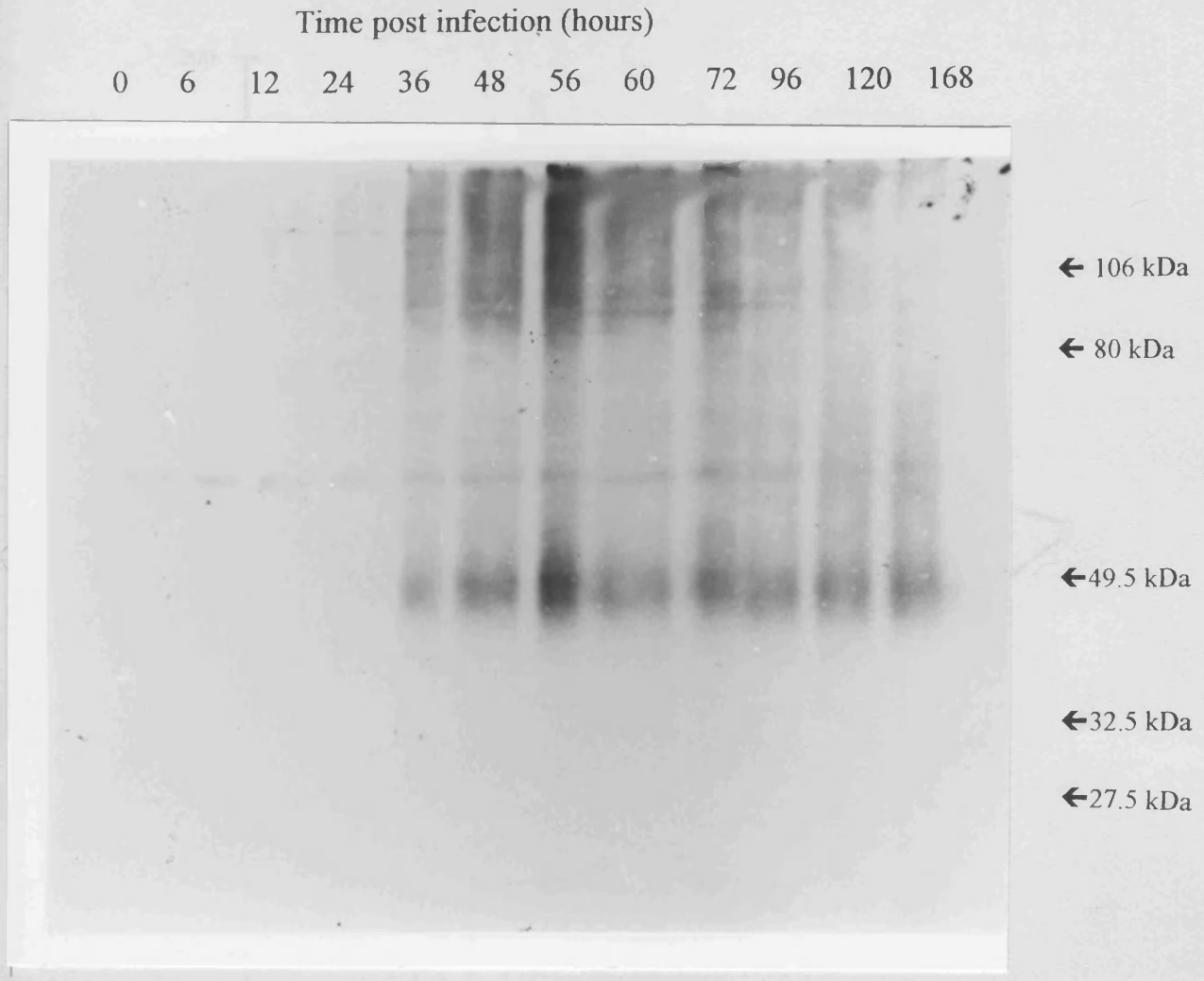


Figure 4.3 Western blotting of the time course of expression of GLUT1 protein produced by the infection of SF9 cells with the recombinant baculovirus E4.2. Cells were infected with a mean of 1.13 pfu/cell. Cell lysates were taken at a range of time points from 0 hpi to 168 hpi (see legend). The 12% SDS-PAG was loaded with a standard volume of sample corresponding to a mean of 20μg total cell protein. The Western blotting was carried out using the anti-C-terminal peptide antibody.

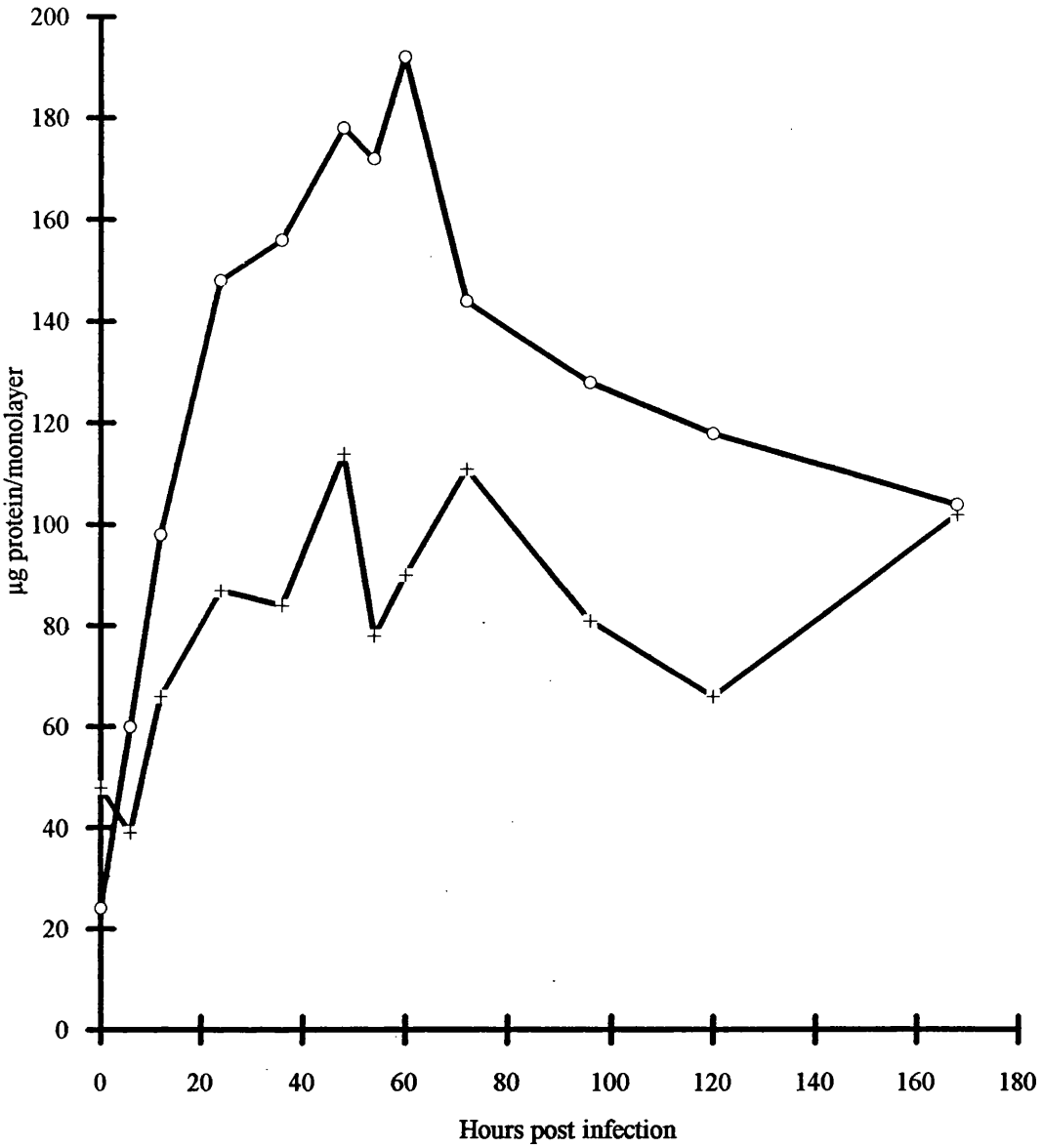


Figure 4.4 Protein estimations of the quantities of protein per 35mm culture dish of Sf9 cells throughout the course of an infection with the recombinant baculovirus E4.2. Total cellular protein (o), and membrane protein quantities (+) were both measured.

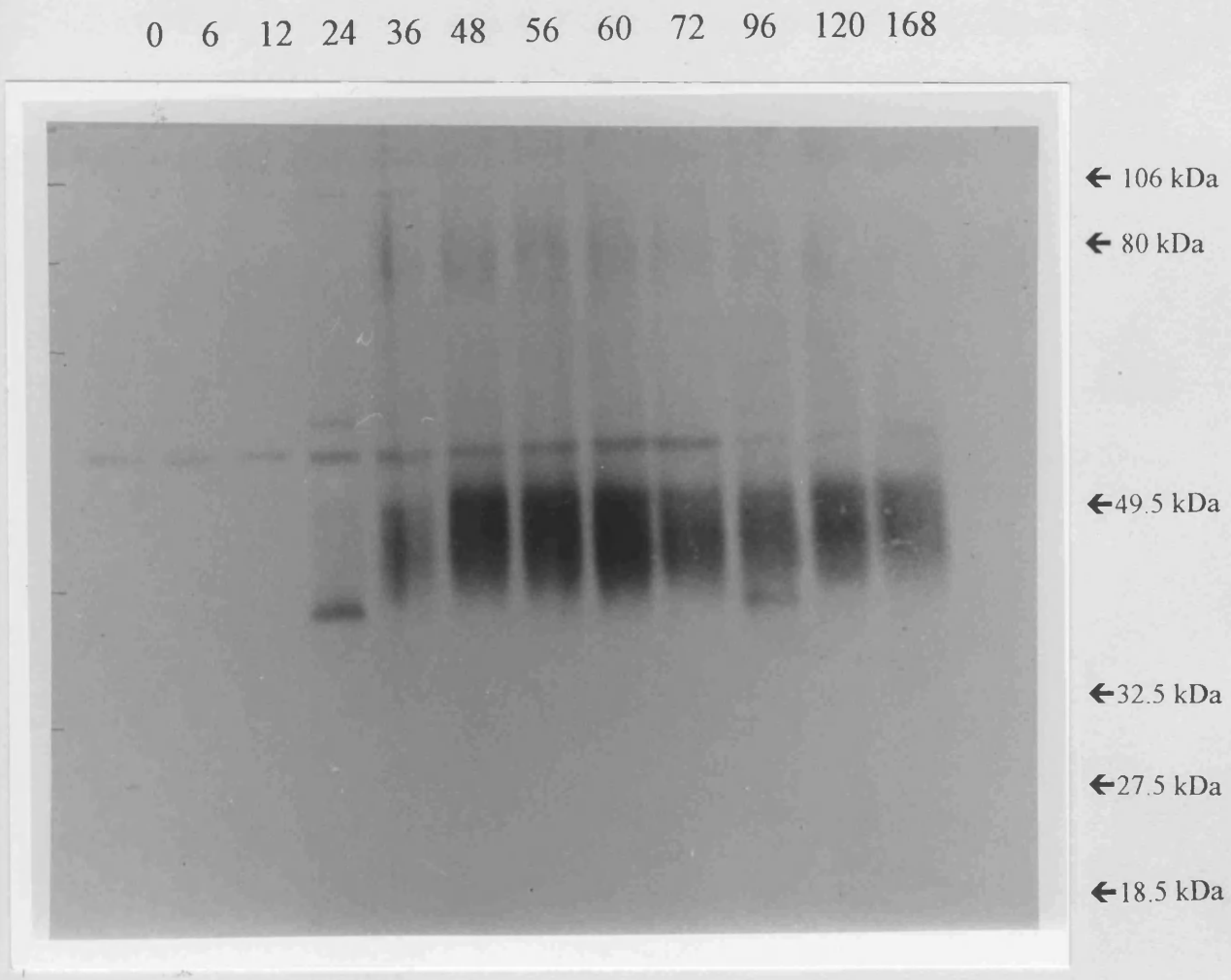


Figure 4.5 Western blotting of membrane samples during the time course of expression of GLUT1 protein produced by the infection of SF9 cells with the recombinant baculovirus E4.2. Cells were infected with a mean of 1.13 pfu/cell. Membrane preparations, as described in section 2.4.1 were made at a range of time points from 0 hpi to 168 hpi. The 12% SDS-PAG was loaded with a standard volume of sample corresponding to a mean of 20μg total cell protein. The Western blotting was carried out using the anti-C-terminal peptide antibody.

relatively healthy during this period, subsequent experiments were carried out at either 48 hpi or 62 hpi on this basis.

4.2 N-TERMINAL HALF PROTEIN

Figure 3.13, in chapter 3, shows the expression of several immunoreactive species by recombinant virus N1, when probed using an antibody directed to the N-terminal half of GLUT1. Figure 4.6 illustrates expression of these species more clearly. The protein migrating at around 31kDa is of the expected size for the N-terminal half protein from the GT78 construct. It is clear from this Western blot that this protein migrates as a doublet, an observation that has been consistently seen on clear Western blots. (this is particularly clear in figure 4.6). In addition to this observation, is the consistent presence of a band at about 52kDa (migrating slightly more slowly than the E4.2 expressed whole GLUT1). Faster migrating species sometimes detected in higher percentage acrylamide gels are considered likely to be degradation products of the higher molecular weight species.

4.2.1 QUANTITATION OF N1 PROTEIN PRODUCT IN INSECT CELLS

Using known concentrations of RBC membranes (therefore known GLUT1 content) as standards, quantitative immunoblotting using the anti N-terminal half anti peptide antibody was used to estimate the relative quantity of immunoreactive GLUT1 species being expressed by cells infected with the N1 virus. Figure 4.6 shows a Western blot used in this quantitation. As is apparent from the blot, there are more immunoreactive species than the expected "N-terminal half" band. Therefore, three figures can be calculated from such an analysis. The quantities of immunoreactive GLUT1 material migrating at the "N-terminal half" monomer molecular weight, material migrating at approximately twice that weight (the putative N-N dimer size), and the total amount of immunoreactive material, including both of these and the higher molecular weight material. Since a monomeric unit is the intended species to be expressed, the first figure is perhaps the most important.

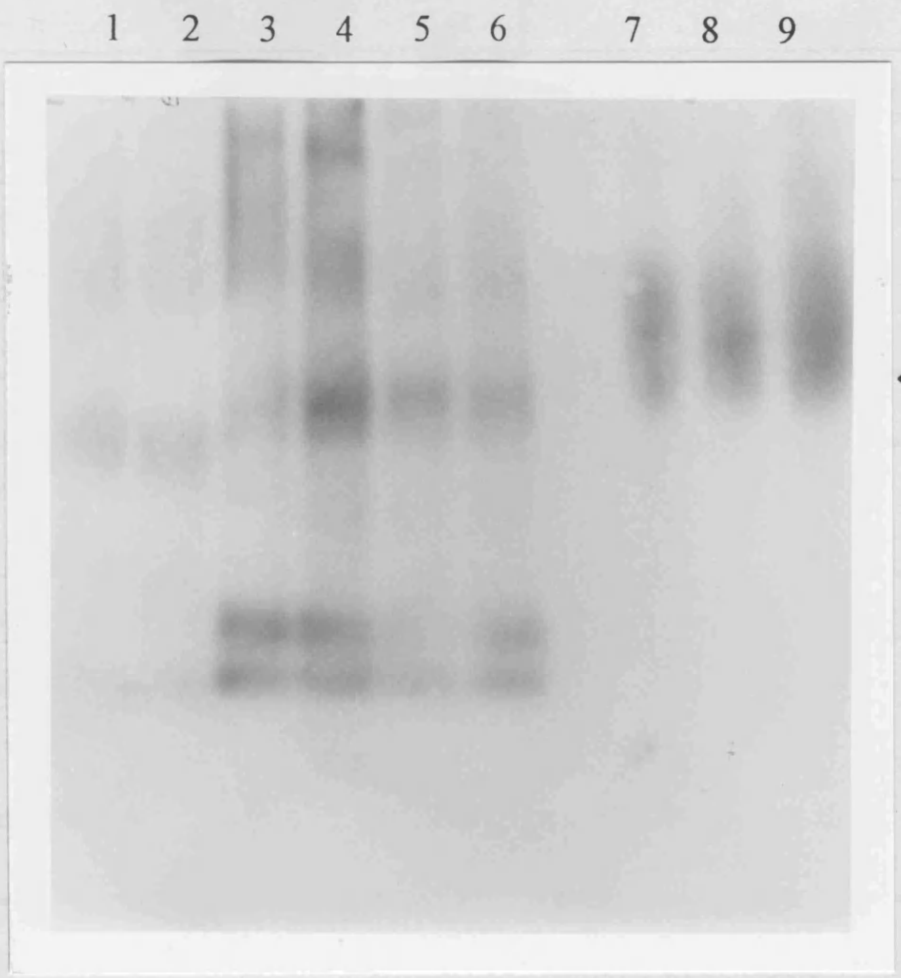


Figure 4.6 Western blot showing samples of Sf9 cells infected with recombinant baculovirus N1, using anti-N-terminal half anti- peptide antibody. Lanes 1 and 2 represent cells infected with virus E4.2, and lanes 7,8 and 9 represent varying quantities of RBC membranes (2, 5 and 10μg protein respectively). Sf9 cell samples infected with virus N1 were prepared as for normal membrane preparations, but centrifuged at $16,000g_{max}$ (lanes 3 and 5). The samples shown in lanes 4 and 6 were prepared as for normal membrane preparations, using centrifugation at $100,000g_{max}$.

From Whole Cell Lysates	
N-terminal half monomer	95.7 pmol/mg
N-terminal putative dimer	30.65 pmol/mg
Total N-terminal immunoreactive material	126.35 pmol/mg

From Membrane Preparation	
N-terminal half monomer	115 pmol/mg
N-terminal putative dimer	91.68 pmol/mg
Total N-terminal immunoreactive material	206.86 pmol/mg

Table 4.3 Quantitation of N1 GLUT1 protein

As is clear from figure 4.6, the Western transfer was apparently not entirely even. This is frequently a problem with this technique, particularly when quantitations are being made from the blot. Apparently (also apparent from the figures in table 4.3) the "dimer" band is in a badly transferred area, particularly on the "whole cell lysates" sample lane. It is deduced from this, therefore, that the figure of 206 pmol/mg membrane protein is probably the more reliable figure. It is clear from a comparison of whole cell lysates against an equivalent quantity of membrane protein that a majority of the protein being expressed is located at the cell surface.

Differential centrifugation of N1-infected cells was carried out, and the expressed protein detected using the anti N-terminal half GLUT1 antibody. The figures shown in table 4.4 are for the relative quantities of N-terminal half "monomer" protein, "dimer" protein, and the total quantities of immunoreactive protein product (not including any lower molecular weight putative degradation products), from centrifugation at 16,000 g_{max} (plasma membranes) as a percentage of product from centrifugation at 100,000 g_{max} (total cellular membranes).

Immunoreactive Species	Percentage at plasma membrane
N-terminal monomer	112%
N-terminal dimer	67.6%
Total N-terminal protein	81.4%

Table 4.4 Cellular localisation of N-terminal protein (virus N1)

These results were found to be reproducible in that all, or at least a great majority of the "monomeric" protein is apparently found to be located at the cell surface, but a significant proportion (approximately 20 to 30%) of higher molecular weight species is apparently localised to intracellular membranes.

4.3 C-TERMINAL HALF PROTEIN.

The expression of protein products from R12 infected cells is demonstrated in the Western blot shown in figure 3.18, and in the figures of section 4.3, probed with the anti GLUT1 C-terminal half antibody. The protein of expected mobility from the construct design is that species seen at an M_r of about 25kDa. As with the expressed N-terminal half (see section 4.2.1), this band is often, though not as clearly, seen as a doublet. The observation of a band appearing at around M_r 50kDa was consistently made in early analyses of the products of this virus, and was the basis of the direction of the second half of the project. This is discussed in chapter six. As with the products of the E4.2 virus and the N1 virus, higher molecular weight species are also almost always observed. Figure 6.4 shows these clearly as discrete bands (also visible in figure 4.9).

4.3.1 QUANTITATION OF R12 PROTEIN PRODUCT IN INSECT CELLS.

C-terminal half protein expressed by cells infected with the R12 virus was quantified by Western blotting with the anti C-terminal half GLUT1 antibody. Note that, as in section 4.2.2, quantities of GLUT1 immunoreactive material in the form of monomeric C-terminal protein, proposed dimeric form, and total immunoreactive material including higher molecular weight material, have been calculated.

From Whole Cell Lysates	
C-terminal half monomer	100 pmol/mg
C-terminal putative dimer	117 pmol/mg
Total C-terminal immunoreactive material	220 pmol/mg

From Membrane Preparation	
C-terminal half monomer	81 pmol/mg
C-terminal putative dimer	128 pmol/mg
Total C-terminal immunoreactive material	221 pmol/mg

Table 4.5 Quantitation of R12 GLUT1 protein

As with the expression of the E4.2 GLUT1-antibody immunoreactive protein products (section 4.1.1), the issue of band intensity and the spread of molecular weights over which a signal is detectable may be important. Although there are three principal immunoreactive species detected in cells infected with the R12 virus, each band is sharp relative to the single immunoreactive GLUT1 species measured in erythrocyte membranes. It is therefore possible that the figures presented in table 4.5 are under-estimates of the expression levels that have been achieved. A visual analysis of almost any Western blot showing both R12 GLUT1 protein and erythrocyte GLUT1

protein indicates relatively comparable band intensities for the erythrocyte GLUT1 and both the monomeric and putative dimeric forms of R12 protein.

In cells infected with the R12 baculovirus, both the R12 protein band (~ 25kDa) and the 50kDa band that are always found in R12 infected cells, are consistently very notably stronger in the membrane samples, than in the whole cell samples. That is , there appears to be more of the expressed protein present as a proportion of the cell membrane protein, than as a proportion of whole cell protein. Since this is clear and reproducible, it seems to be an indication of membrane insertion of the expressed R12 protein. (see figure 4.7). In order to formally determine the cellular localisation of C-terminal half GLUT1 protein (R12 virus), differential centrifugation was carried out. R12 infected cells were harvested at 48 hpi and the membrane preparation protocol was followed to just prior to centrifugation (2.2.3.1). The sample was then halved, and one half subjected to centrifugation at 16,000 g_{max} (plasma membranes), the other 100,000 g_{max} (total membranes). The results of a representative differential centrifugation experiment are shown in table 4.6.

Immunoreactive Species	Percentage at plasma membrane (16,000 g/ 100,000g)
C-terminal "monomer"	89.4%
C-terminal "dimer"	136%
Total C-terminal GLUT1 protein	117%

Table 4.6 Differential centrifugation of R12 infected cells.

It is clear from table 4.6 that most of the R12-expressed C-terminal GLUT1 protein is expressed at the plasma membrane of the insect cells. It is interesting to note that, where only around 90% of the C-terminal monomer

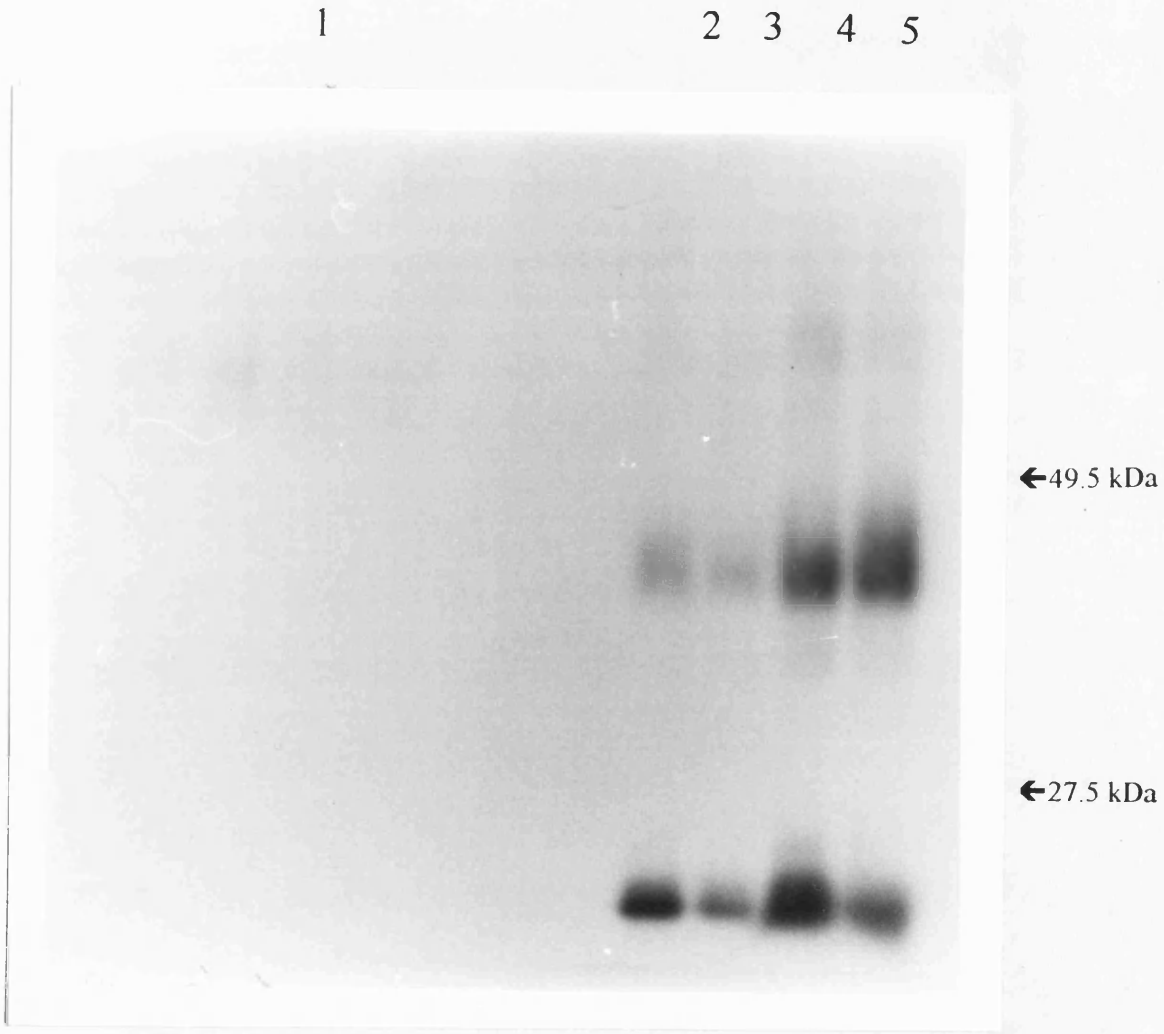


Figure 4.7 Western blotting of Sf9 cell lysates (lanes 3 and 5) and membrane preparations (lanes 2 and 4) of cells infected with the recombinant baculovirus R12. Cells were infected with 1.66 pfu/cell, and lysates or membranes were made at 48 hpi. Small samples were taken for protein estimation, and 15μg protein per sample was loaded onto the 10% SDS-PAG. The Western blot was carried out using the anti-C-terminal peptide antibody. Lanes 1 represents red blood cell samples used in the quantitation.

protein is apparently present at the cytoplasmic membrane in cells infected only with the R12 virus, when cells are dually infected with the R12 virus and the N1 virus, the figure calculated for the percentage of "R12 monomer" present in the plasma membrane is 133%.

4.3.3 DETERMINATION OF OPTIMAL PROTEIN EXPRESSION

In order to determine the best possible balance between expression levels and cell deterioration, a full time course was carried out on cells infected with the R12 virus. Table 4.7 gives a summary of the timepoints used, with brief descriptions of the appearance of the cells at landmark timepoints. The progression of the infection followed an expected course, with cells gradually showing deterioration from about 48 hpi onwards, to an estimated 10% of cells still alive at 120 hpi. Cells infected with AcNPV:LacZ (LZ) DNA followed a similar course, demonstrating the recombinant virus infection to be fairly typical. Mock infected cells observed over the same period showed normal growth, to confluence at between 72 and 96 hpi, with the first signs of over-confluence (cells becoming "sick" in appearance, and floating off the plastic surface) at the last timepoint, 120 hpi.(summarised in table 4.7). Figure 4.8 is a graph of the total amounts of protein in the R12 infected cells (estimated using the BCA method, see 2.2.3.9), and demonstrates the early growth of the monolayer, which appears to slow rapidly, followed by a reduction in protein as the cells begin to die. (This is not intended to give any indication about the quantities of GLUT1 protein being expressed).

Figure 4.9 is a Western blot (using the anti C-terminal half Glut1 antibody) of time course samples. An estimated 20mg per sample was used, and this blot therefore demonstrates visually the quantities of GLUT1 protein present relative to the quantity of total protein, at each time point. It is clear from this that GLUT1 C-terminal half protein is not detectable before the 24 hpi time point. There appears to be no appreciable increase in GLUT1

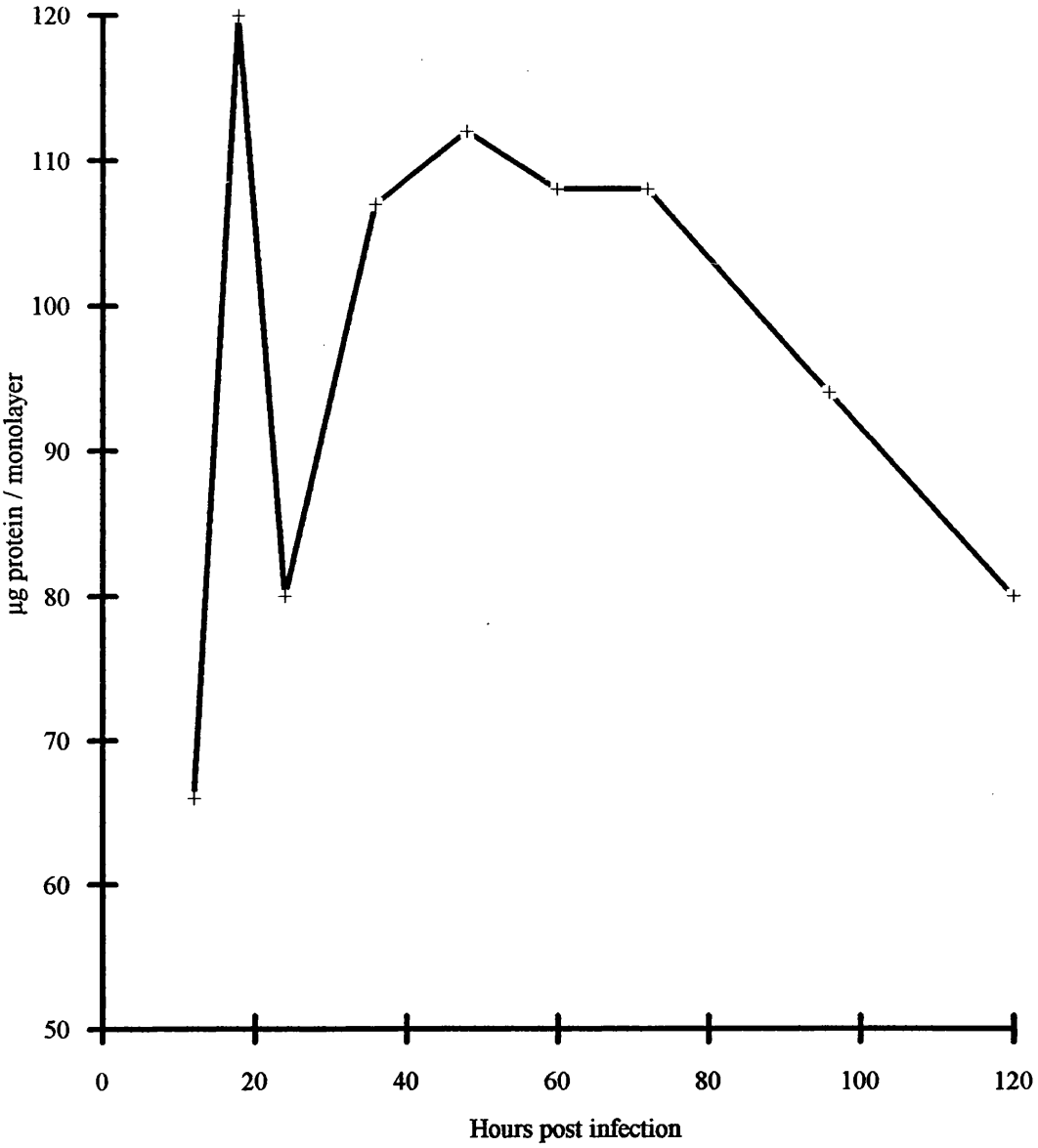


Figure 4.8 Protein estimations of the total amount of protein per 35mm culture dish of Sf9 cells throughout the course of an infection with the recombinant baculovirus R12.

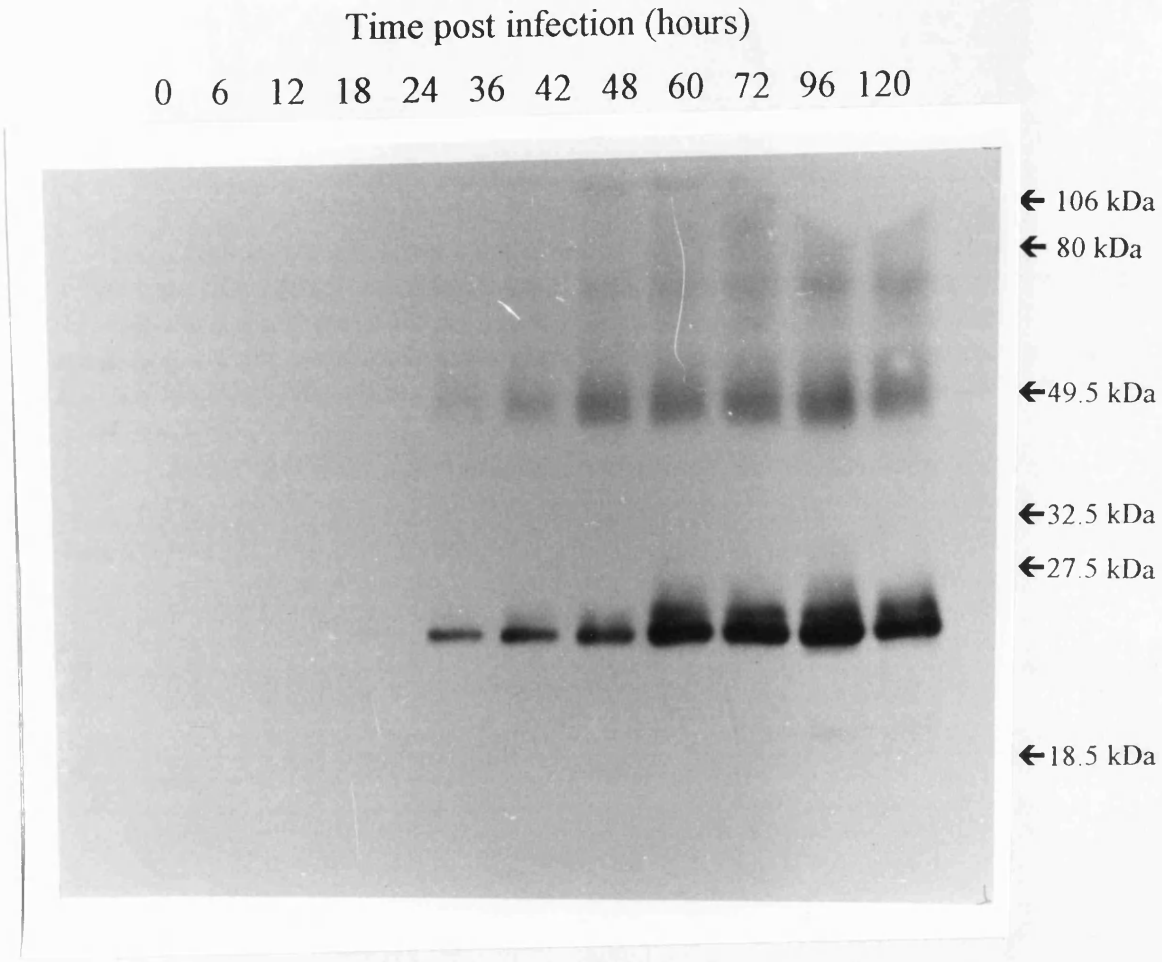


Figure 4.9 Western blotting of the time course of expression of anti-GLUT1 C-terminal peptide antibody immunoreactive protein produced by the infection of Sf9 cells with the recombinant baculovirus R12. Cells were infected with a mean of 1.67 pfu/cell. Cell lysates were taken at a range of time points from 0 hpi to 120 hpi. Protein estimations were made of each sample, and the 10% SDS-PAG was loaded with a standard 20μg protein per sample.

TIME POST INFECTION (HOURS)	MAIN VISUAL FEATURES R12 infected cells	Control "LZ" infected cells	Control "mock" infected cells
0	Cells infected at time zero		
6			
12			
18			
24	cells possibly less dense than mock infected cells ~20 to 30% confluent	~20 to 30% confluent	~20 to 30% confluent
36			
42			
48	~30 to 40% confluent. Some cells beginning to appear sick, reduced growth rate very apparent	~50 to 60% confluent. Some cells beginning to appear sick	~80% confluent. All cells appear healthy.

60			
72	30 to 40% confluent. ~50% cells floating, of the remainder, ~ 40 to 60% appear sick	~80% confluent. ~20% floating, ~40% appear sick	>90% confluent. ~2% cells floating, but majority of remainder still appear healthy
96	~40 to 50% cells floating. Of remainder, only occasional cells seem healthy. Most notable feature being very low total cell numbers	>30% of cells floating. of the remainder, only ~15% appear healthy	~90% confluent. ~2% cells floating. A few cells beginning to show signs of deterioration
120	~10% confluence. >50% cells floating. No evidence of healthy cells. Cell mass on centrifugation ~50% of mock infected cells.	~50% confluent. ~ 30% cells floating. ~10% of remaining cells still appear healthy	>90% confluent. ~10% cells floating. Monolayer appears typically "old"

Table 4.7 Visual record of the time course of Sf9 cell baculovirus infection.

production after about sixty hours, and that, as a proportion of the total amount of protein remaining, GLUT1 is not reduced as the cells begin to die.

Figure 4.10 is a Western blot where a standard volume of material has been used per sample (30μl, corresponding to a mean of 30μg), and therefore demonstrates the overall quantities of GLUT1 material at each timepoint, relative to the number of cells infected (i.e. rather than as a proportion of the total cell protein). From this, there is no discernible signal of GLUT1 protein until 36 hpi, and reflects the observation that the cells are still growing at this stage. There also appears to be an ultimate reduction in quantity at the later stages of the infection, presumably reflecting the observed cell death. From this, an optimum expression level of between 48 and 72 hpi can be determined.

In order to gain a clearer view of expression in relation to infection state, a further analysis of these samples was made. Quantitative immunoblotting was carried out on gels loaded with a constant volume (20μl, corresponding to an estimated 20μg) of each sample. Figure 4.11 is the Western blot, and figure 4.12 shows the calculated relative quantities of both the C-terminal half monomer species, and the "dimer" band (as measured in cpm of measured radioactivity content of isolated bands on the blot.). From this, 60 hpi appears to be a clear optimum expression time for this protein. However, in view of the fact that the cells are in a fairly advanced stage of infection, and are beginning to deteriorate significantly by this stage, 48 hpi was taken to be a better time to use cells for further experiments, since the cells are still relatively healthy, but producing plenty of protein. The relative expression of the R12 putative dimer band is also shown on this graph.

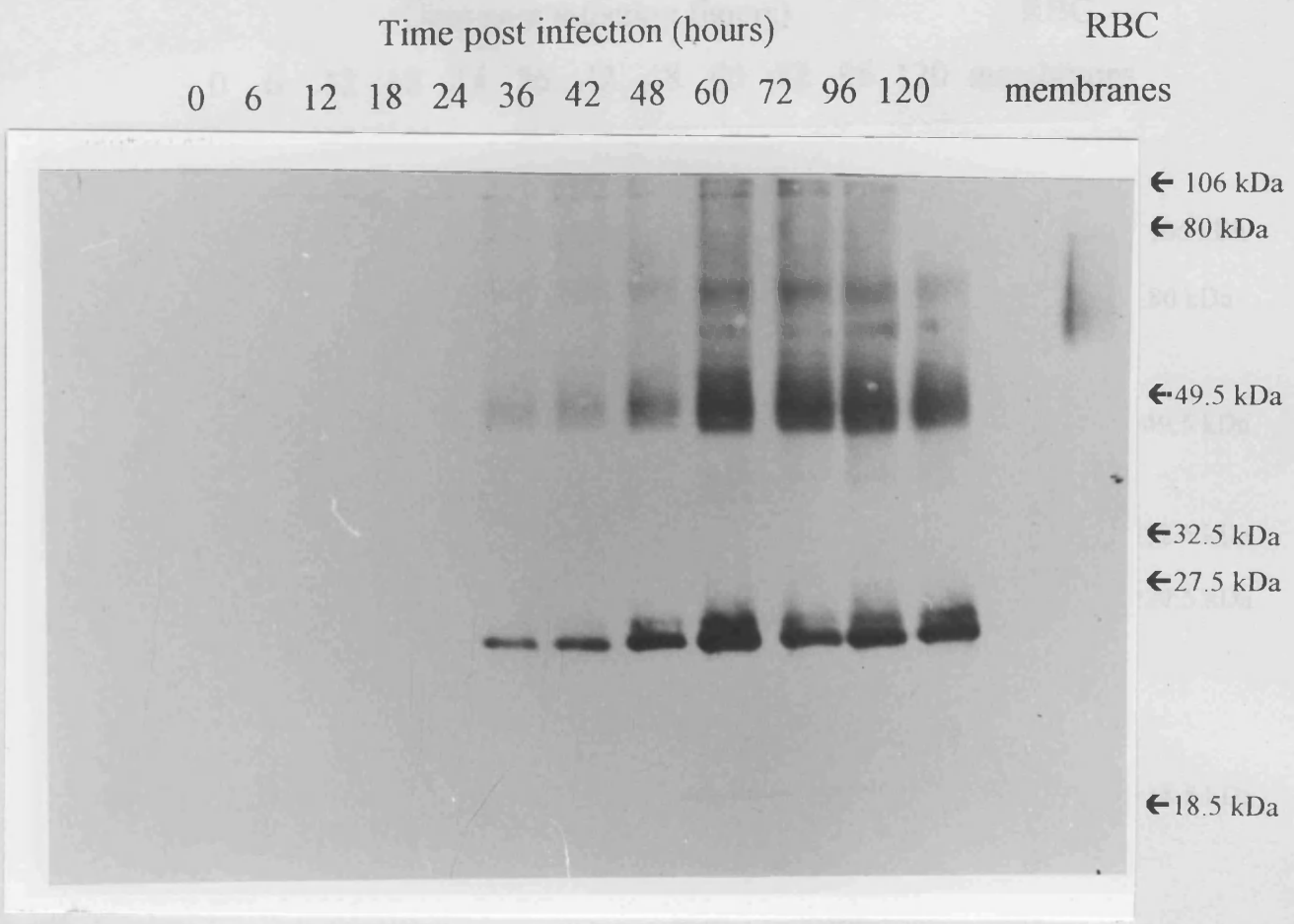


Figure 4.10 Western blotting of the time course of expression of anti-GLUT1 C-terminal peptide antibody immunoreactive protein produced by the infection of Sf9 cells with the recombinant baculovirus R12. Cells were infected with a mean of 1.66 pfu/cell. Cell lysates were taken at a range of time points from 0 to 120 hpi. The 10% SDS-PAG was loaded with a standard volume of sample corresponding to a mean of 30μg total cell protein.

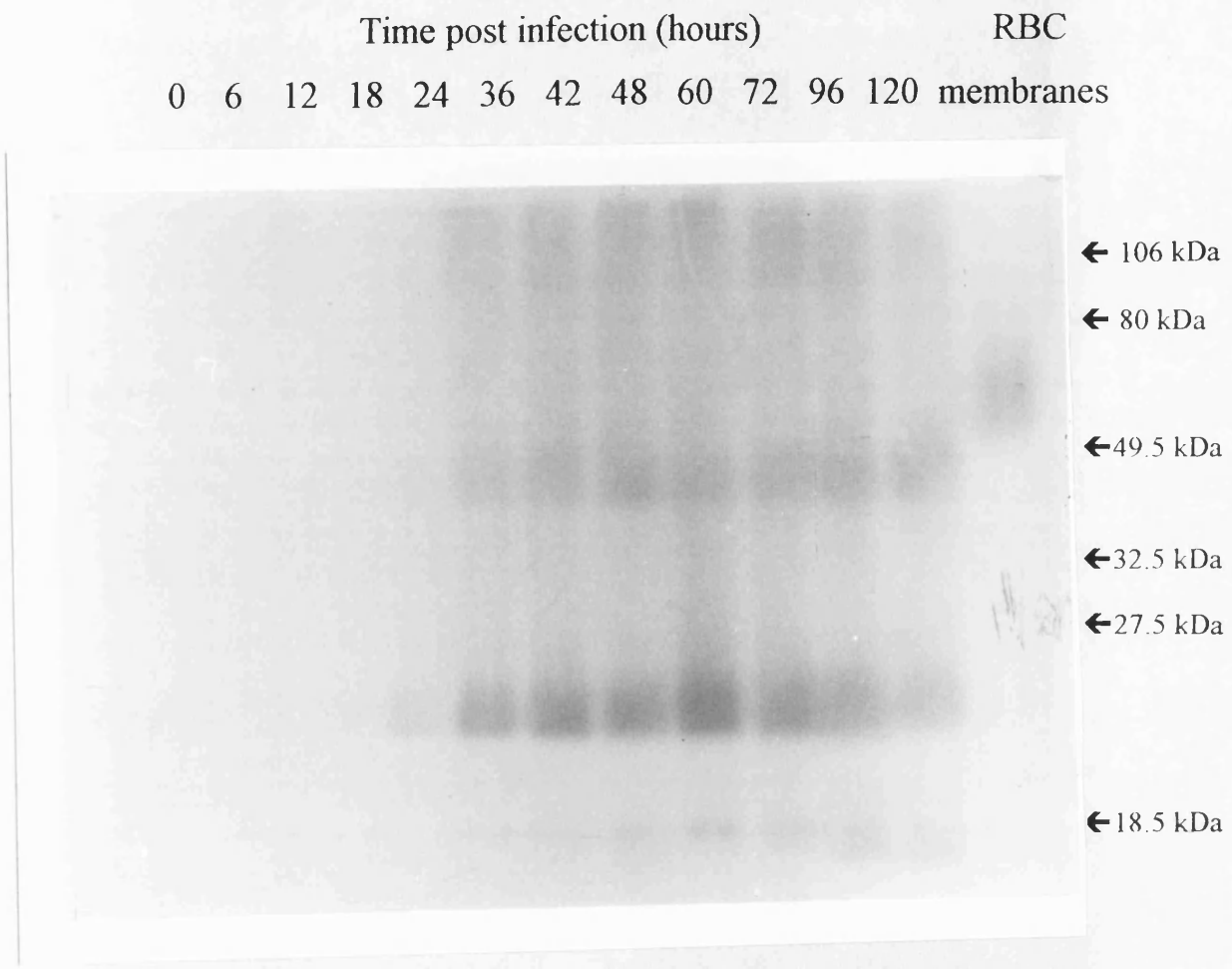


Figure 4.11 Quantitative Western blotting (see section 3.4.4) of the time course of expression of anti-GLUT1 C-terminal peptide antibody immunoreactive protein produced by the infection of Sf9 cells with the recombinant baculovirus R12. Cells were infected with a mean of 1.66 pfu/cell. Cell lysates were taken at a range of time points from 0 hpi to 120 hpi. The 10% SDS-PAG was loaded with a standard volume of sample corresponding to a mean of 30μg total cell protein.

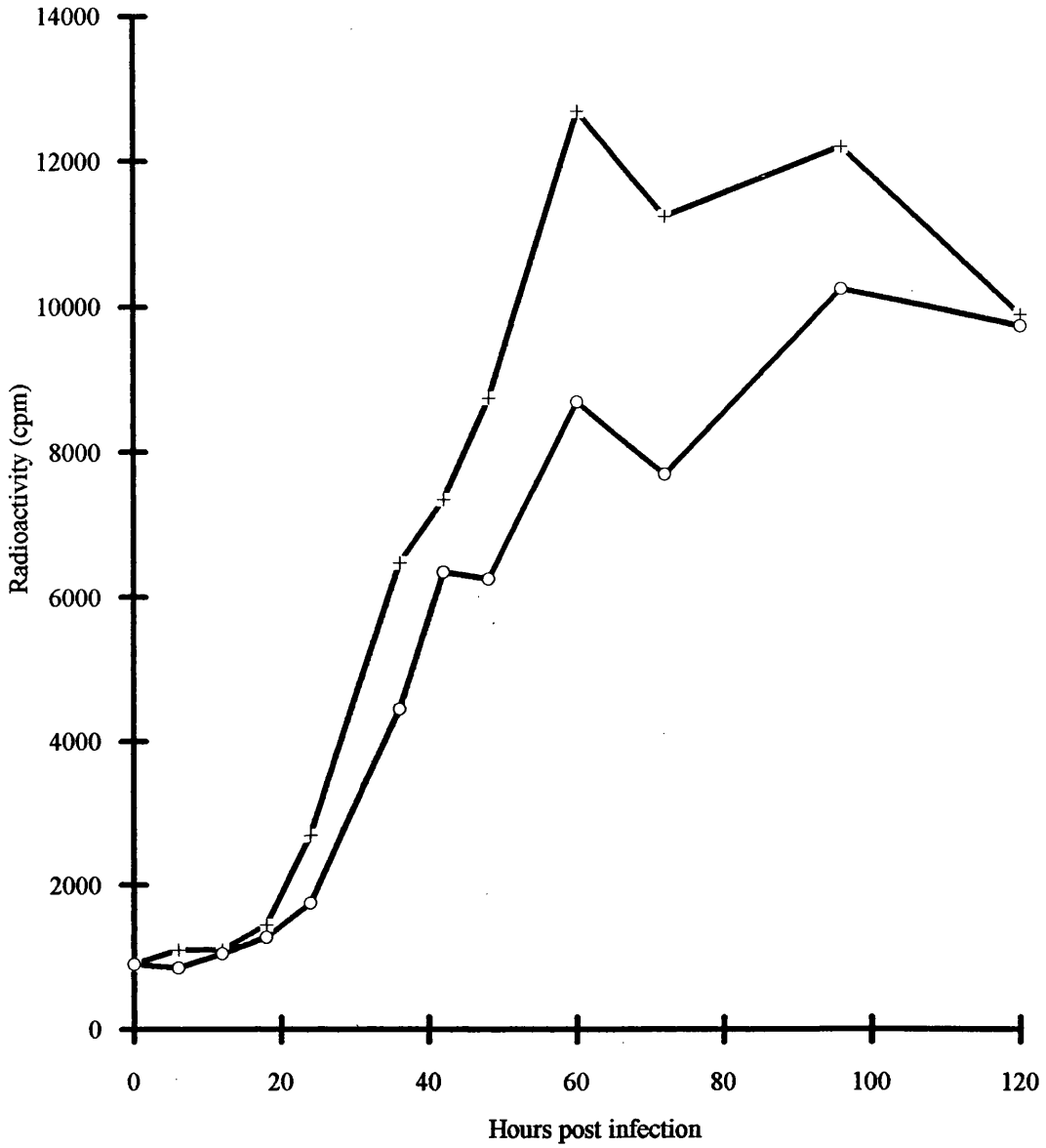


Figure 4.12 Relative quantities of Sf9cell/R12-expressed GLUT1 antibody immunoreactive protein over the course of a viral infection. The samples were prepared as described in section 2.2.3.5, and quantitated by quantitative immunoblotting (section 2.2.3.4, see figure 4.11). Time course of expression of both R12 C-terminal GLUT1 "monomer" (+) and "dimer" (o) species are shown.

4.4 DISCUSSION .

In summary, Western blotting, using the two anti-GLUT1 antibodies, has identified the successful expression of GLUT1 protein in the form of the whole protein, the N-terminal half, and the C-terminal half (Western blotting profiles of each of the products can be seen in sections 4.1, 4.2 and 4.3). Comparisons of the main products can be made from figure 4.13. Their insertion into the membrane appears to be successful, even in the C-terminal half of the protein, which may not necessarily be expected to have any insertion signal sequence. The expression levels attained for each protein are within an order of magnitude of native expression of GLUT1 in human erythrocytes.

The expressed full-length protein has a greater mobility on SDS-PAGE than its red blood cell counterpart, which may reflect its glycosylation state when expressed in insect cells, as being incomplete as regards the transporter in erythrocytes. This is discussed in full in chapter seven.

The sizes of the DNA constructs for the N- and C-terminal halves are very similar, at 740 and 810 bp respectively; corresponding to 246 and 267 amino acids of translated protein. Considering only the polypeptide chain, the products would be expected to vary in weight by no more than two and a half kDa, both being between 25 and 27.5 kDa. As this would predict, the C-terminal product of the R12 virus runs at a mobility of ~25kDa. The N-terminal protein, however, has a mobility of about 31kDa, and this is likely to reflect some degree of glycosylation, at the single GLUT1 glycosylation site, Asn 45. That this product migrates as a doublet supports this, by suggesting a degree of heterology of the glycosylation.

The appearance of the "dimer" bands for the two half-GLUT1 proteins, and the observation of higher molecular weight species of all products, are discussed in chapters six and seven.

If a comparison is made of the quantitation figures for each of the protein products, from whole cell lysates and membrane preparations, it is very clear that there is very little difference between the two. That these figures are so similar is obviously indicative of localisation of the proteins in the membranes of the cell. The very close similarity of these figures may be indicative of a cellular change occurring in response to the expression of membrane-associated proteins. That is, in order to accommodate the large quantities of membrane protein present, more membrane, presumably all components, may be manufactured by the cell. In order to investigate this simply, a series of very carefully carried out protein estimations were made on a series of cells.

Cells, in 35mm dishes were infected (as described in chapter 2) with either a "mock" infection, "LZ" baculovirus (the product of which is cytoplasmically targeted); or one of the three recombinant baculoviruses, E4.2, N1, or R12. After 48 hours the cells were scraped from the dishes (as described in section 2.2.3.1). The sample volume was measured, and a large sample was taken for protein estimation. The remainder was prepared as membranes using the usual procedure. A large sample of the membrane preparation samples was taken for protein estimation. Large samples were also taken of the membrane preparation supernatant, for protein estimation. The results are presented as percentages of total cellular protein (from the first set of estimation samples taken) present in the membrane (100,000 g_{max} pellet) and non-membrane (100,000 g_{max} supernatant) fractions of the insect cells. The "total" column gives an indication of the efficiency of the protein estimations, and inaccuracies may also indicate loss of material during the procedure.

Sample	% protein in membrane	% protein in non-membrane	(total)
mock	62	42	104
LZ	57	28	85
E4.2	80	11	91
N1	128	(too low)	-
R12	97	2.5	99.5

Table 4.8 Relative protein quantities in cellular fractions

It is quite clear from this table, particularly from the R12 data which appears to be the most accurate, that a larger proportion of the total cell protein is indeed being incorporated into the membranes of the cell in response to the expression of membrane proteins encoded by the recombinant baculoviruses.

CHAPTER 5

FUNCTIONAL STUDIES

5.1 CYTOCHALASIN B

5.1.1 INTRODUCTION

Cytochalasin B, a fungal metabolite, specifically and reversibly binds to the internal glucose binding site of GLUT 1 (Helgerson and Carruthers, 1986). It had been used as a potent inhibitor of glucose transport in human erythrocytes even before the binding protein had been characterised or identified (Taverna and Langdon, 1973 and Lin and Spudich 1974). Three cytochalasin B binding sites were identified in erythrocytes, but it was found that only one type was glucose sensitive, and that this represents over 60% of the total cytochalasin B binding sites. This one type was also found to be the only one of the three that does not also bind cytochalasin E (Jung et al, 1980). Taverna and Langdon (1973) suggested that the protein to which cytochalasin B binds, and the glucose transporter may be the same protein. In 1974, Lin and Spudich found that the ability of a sugar to inhibit the binding of cytochalasin B to erythrocytes was related to their affinity for the sugar transport system. This was supported by Kasahara and Hinkle (1977), and by Hinkle et al (1979) , who reconstituted erythrocyte proteins into liposomes. Significant transport function and significant cytochalasin B binding were both found in this system. The only protein found to be sufficiently abundant to account for either of these findings was a protein appearing as a broad band around 55 kDa molecular weight on SDS-PAGE. Many later workers have shown cytochalasin B to bind the glucose transport protein, and the specific localisation of its binding has been closely determined.

Red blood cells are impermeant to cytochalasin B. It is thought to bind at the internal glucose binding site, and so it is used as a competitive inhibitor of glucose binding at, or very near to, the internal site. It is therefore a competitive inhibitor of glucose efflux, and a non-competitive inhibitor of

glucose influx across the plasma membrane. The molecular structure of cytochalasin B is illustrated in figure 5.1 .

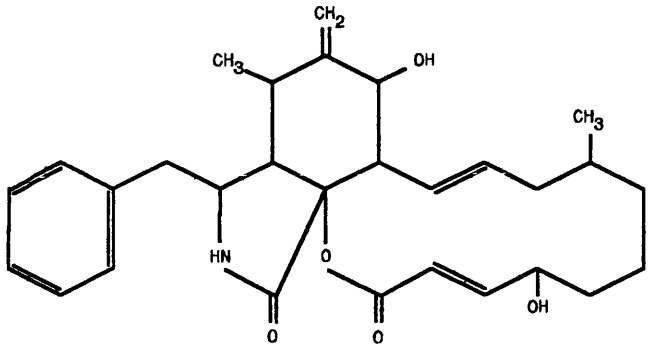


Figure 5.1 Cytochalasin B

However, its use as an affinity ligand was initially limited by the reversible nature of its binding. In 1981 and 1982, Carter-Su et al, and Shanahan independently discovered that cytochalasin B is a natural photoaffinity ligand, becoming a highly reactive molecule upon exposure to high intensity ultraviolet irradiation. The nature of the covalent labelling of the glucose transporter with cytochalasin B is now thought to be by the photoactivation of the transporter protein (Cairns et al, 1987). The use of [^{3}H] cytochalasin B to covalently label the glucose transporter, GLUT 1, of human erythrocytes or erythrocyte membranes is now an established method. Labelling is inhibited by the presence of D-glucose (Carter-Su et al recorded an average reduction of binding of the major binding peak of 72% +/- 5% in the presence of 500mM D-glucose). GLUT 1 in red blood cells is covalently labelled with tritiated cytochalasin B as a broad peak over the molecular weight range 45 to 65 kDa, peaking at around 55 kDa, (Carter-Su et al, 1981) which is the range covered by the transporter when analysed by Western blotting with a specific antibody. Cytochalasin B labelling of erythrocyte membranes was

carried out, and is shown in Figure 5.2. Western blotting of erythrocyte membranes, using a specific anti-GLUT 1 antibody is shown in figure 3.4.

The aim of cytochalasin B labelling experiments on the transporter proteins expressed in insect cells is to determine the presence of a conformationally functional cytochalasin B binding site, and to determine whether any cytochalasin B labelling that does occur is displaceable by glucose. That is, to determine the presence of a functional cytoplasmic-side glucose binding site, in these expressed proteins.

5.1.2. CYTOCHALASIN B LABELLING EXPERIMENTS ON EXPRESSED FULL-LENGTH PROTEIN (E4.2)

It would be anticipated that the full length protein, having been demonstrated to be membrane associated, would be in an appropriate conformation, and therefore possess functionally intact glucose binding sites. From its observed molecular weight, the expressed glucose transporter would be expected to demonstrate a corresponding cytochalasin B labelling profile. That is, the peak would be expected to be slightly narrower than that observed of GLUT 1 in red blood cells (RBC), and at a M_r of ~50 kDa, rather than the 55 to 65 kDa of RBC.

Labelled cells were immunoprecipitated with the anti GLUT 1 antiserum in order to improve the specificity and clarity of the results.

A number of labelling experiments were carried out on uninfected Sf9 cells, and cells infected with "wildtype" (AcNPV:LacZ) baculovirus, to determine whether any indigenous insect hexose transporter would be labelled with this ligand. Cytochalasin B inhibition of glucose transport has recently been demonstrated in *Drosophila melanogaster* K_c cells (Wang and Wang, 1993). However, they also demonstrated that, by using full- length cDNA clones of the mammalian transporters, homologous DNA could not be found in these cells despite the cytochalasin B inhibition results. Figure 5.3 shows

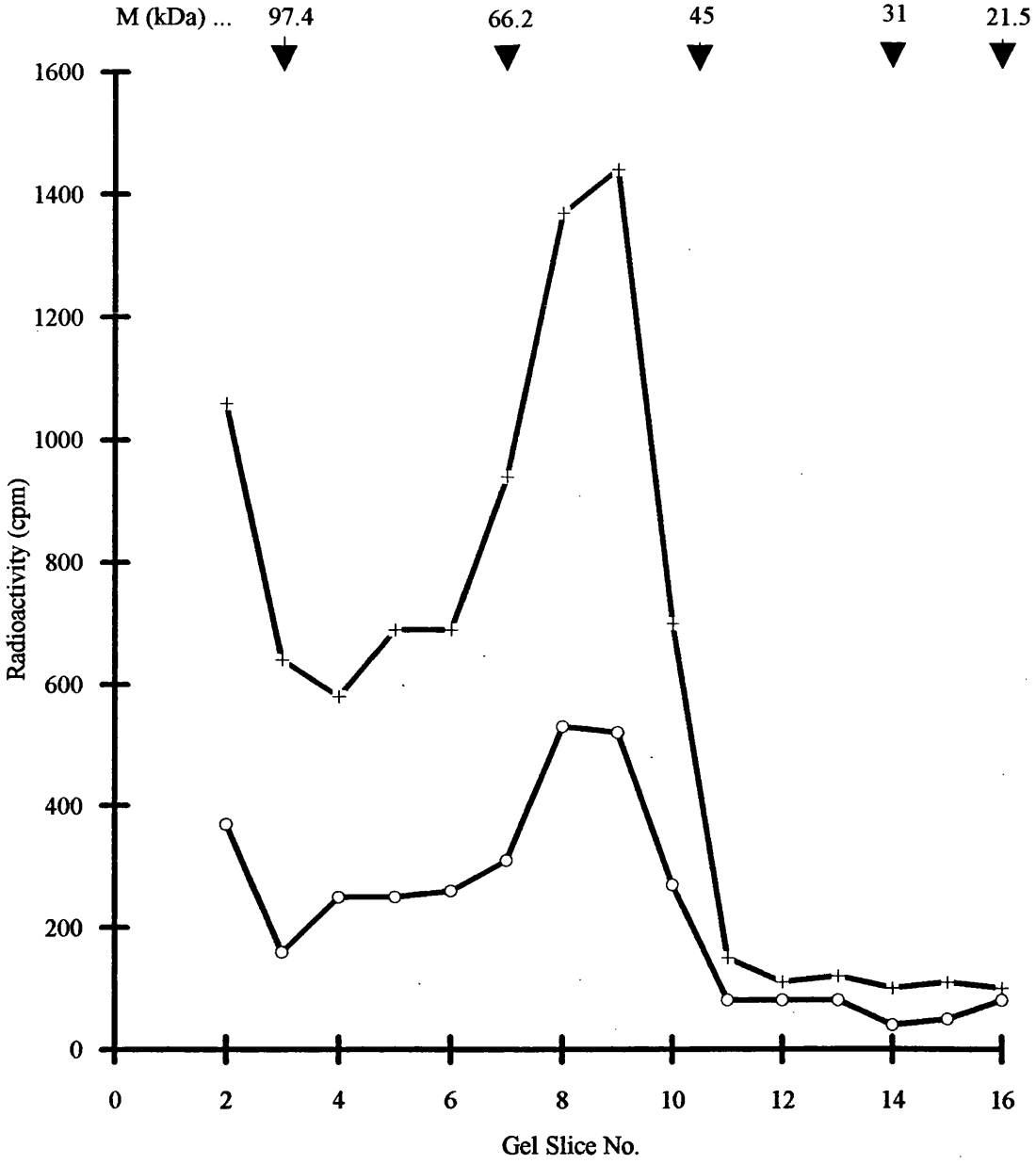


Figure 5.2 Cytochalasin B labelling of erythrocyte membrane GLUT1. Membranes were incubated with cytochalasin B in the presence (o) and absence (+) of 200mM D-glucose, and irradiated using a Rayonett photoreactor. The solubilised membranes were immunoprecipitated using the anti- C-terminal GLUT1 antiserum (see 2.2.3.6 and 2.2.3.7). The labelling peak at ~55 kDa represents the cytochalasin B labelled GLUT1 protein.

profiles for cells infected with the wildtype virus (encoding polyhedrin), and the "wildtype" AcMNPV:LacZ (as used in co-transfections), and demonstrates that Sf 9 cells infected with baculovirus (ie. not baculovirus encoding glucose transporter protein) do not naturally express proteins that are both labelled with cytochalasin B and immunoreactive with the anti GLUT 1 antiserum.

Figure 5.4 shows [^{3}H]-cytochalasin B labelling of Sf9 cell membranes expressing the E4.2 full length glucose transport protein. This quite clearly demonstrates that all of the predictions were accurate. The labelling peak is clear, and at a molecular weight of ~50 kDa. On the addition of D-glucose to the same experimental condition, the peak is almost entirely eliminated, demonstrating the inhibition of labelling by glucose.

Figure 5.5 represents concomitantly labelled RBC membranes and E4.2- infected Sf9 cell membranes. This illustrates the molecular weight difference of the cytochalasin B labelling species between the two, which appears to be the same as the difference observed in Western blotting with the anti- GLUT 1 antibody used in the immunoprecipitation.

Figure 5.5 also clearly illustrates a height difference between the RBC and insect cell expressed GLUT 1 labelling peaks. Although expression levels for the protein expressed in Sf9 cells had not been determined before these experiments, approximately matched transporter quantities had been used in both conditions (as determined visually by the intensity of immunoreactivity on Western blotting). Differences in immunoreactive GLUT 1 presence would therefore seem unlikely to account entirely for the difference in cytochalasin B (and therefore glucose) binding, and the difference is therefore more likely to be due to either a difference in binding affinity, or reflective of the fact that there may be immunogenic but non-functional transporters being expressed as well as functional ones. This is discussed in chapter 7.

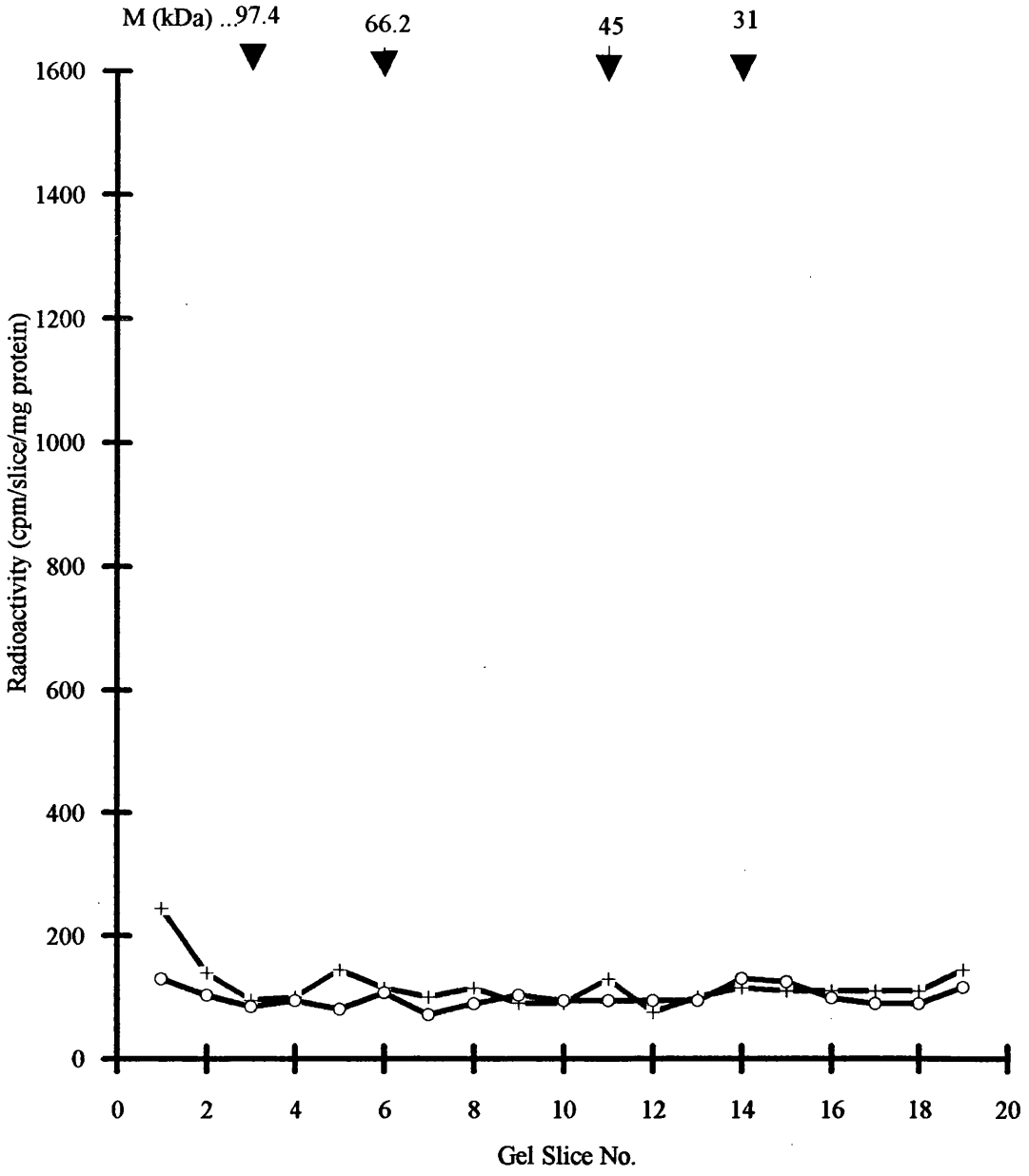


Figure 5.3 Cytochalasin B labelling of membranes of Sf9 cells infected with "wildtype" baculovirus (AcMNPV) (o); and cells infected with AcMNPV:LacZ type "wildtype" baculovirus (+). Membranes were prepared (as section 2.2.3.1), labelled with cytochalasin B (2.2.3.7), and immunoprecipitated using the anti-C-terminal GLUT1 antiserum (2.2.3.7). Immunoprecipitated samples were subjected to a 12% SDS-PAGE. No distinct labelling peaks are discernible for either sample.

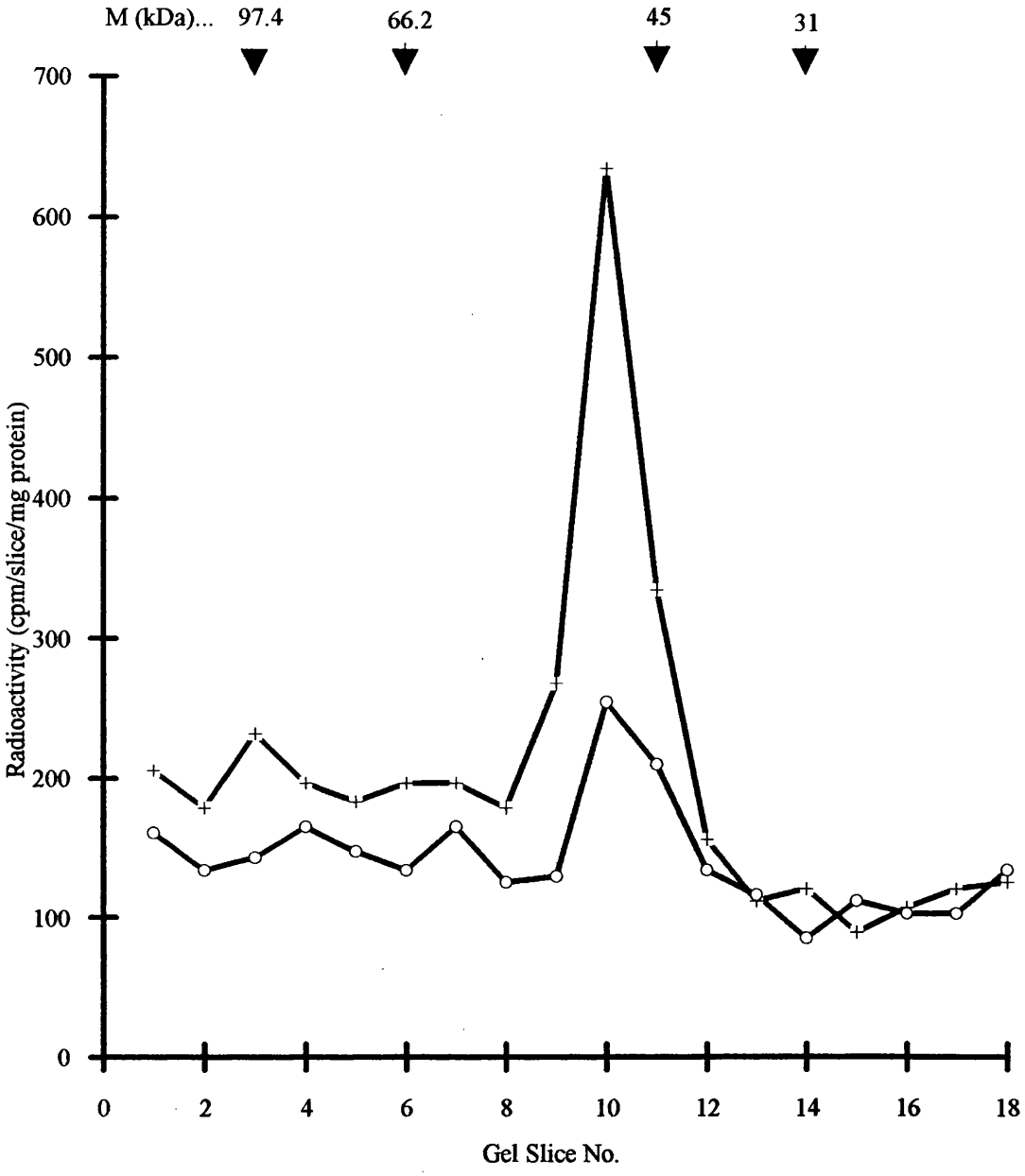


Figure 5.4 Cytochalasin B labelling of membranes of Sf9 cells infected with recombinant baculovirus E4.2, encoding the full-length GLUT1 protein, in the presence (o) and absence (+) of 200mM D-glucose. Labelled membranes (2.2.3.7) were solubilised, and immunoprecipitated using the anti C-terminal GLUT1 antibody (2.2.3.6). The major labelling peak migrated at a molecular weight of ~50 kDa; and is shown to be inhibited by the presence of D-glucose.

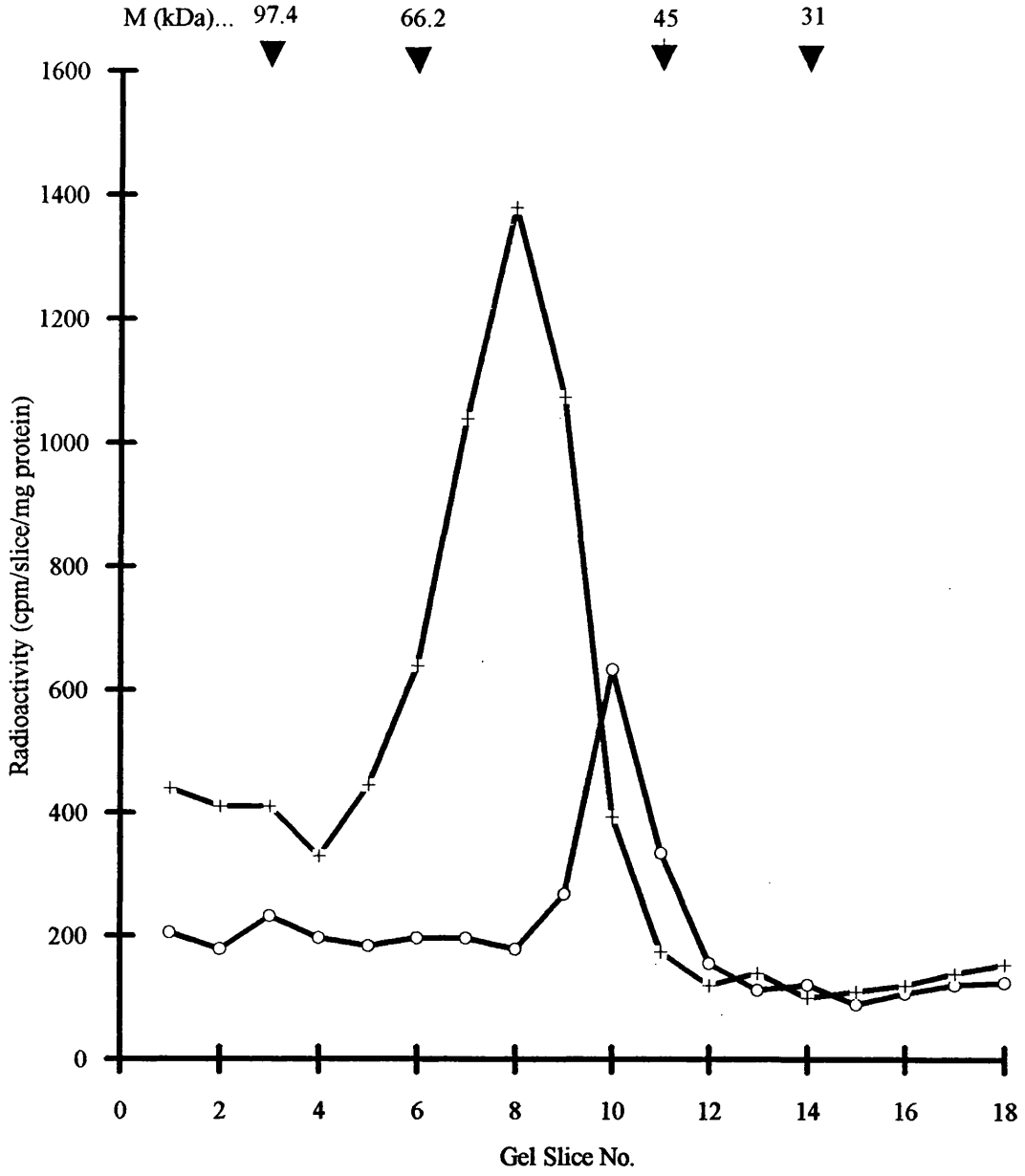


Figure 5.5 Cytochalasin B labelling of erythrocyte GLUT1 compared to Sf9/E4.2 expressed GLUT1. Cell membranes were labelled with cytochalasin B (as described in 2.2.3.7) and immunoprecipitated using the anti C-terminal GLUT1 antibody. The RBC (+) and E4.2 (o) conditions were carried out simultaneously and subjected to SDS-PAGE on a 12% acrylamide gel. The mobility difference on SDS-PAGE between the two GLUT1 proteins is clear.

5.1.3 CYTOCHALASIN B LABELLING EXPERIMENTS ON N-TERMINAL HALF PROTEIN (N1)

There has never been any suggestion for the localisation of the cytoplasmic glucose binding site at any point on the N-terminal half of the GLUT 1 glucose transport protein. Indeed, there is evidence to demonstrate that cytochalasin B labelling is NOT associated with the N-terminal half of the protein (Cairns et al, 1987; Davies et al, 1987; Karim et al, 1987). However, since the whole transporter expressed in Sf9 cells has been demonstrated to bind cytochalasin B, the demonstration of non-labelling of the similarly expressed independent N-terminal half was considered to be important.

Figure 5.6 shows labelling profiles for N1-infected cells expressing the N-terminal half protein. These membrane samples were immunoprecipitated using the anti N-terminal GLUT 1 antiserum (Western blots using this antibody to detect the expression of RBC GLUT 1, E4.2 protein, and N1 protein can be seen in figure 3.12 in chapter 3).

This clearly shows that no labelling can be detected. Since no labelling was detectable, the addition of glucose to the experimental condition was not performed.

5.1.4 CYTOCHALASIN B LABELLING EXPERIMENTS ON THE C-TERMINAL HALF PROTEIN

There is a large body of evidence placing the location of the cytoplasmic-side glucose binding site on the C-terminal portion of the transporter protein. For example, Davies et al (1987) and Holman and Rees (1987) showed that tryptic digests of GLUT 1 protein yield two major fragments, one of around 18 kDa, and a glycosylated fragment of around 22 kDa. Labelling the glucose transporter with cytochalasin B before digestion showed that the cytochalasin B binds to the 18 kDa fragment. Amino acid

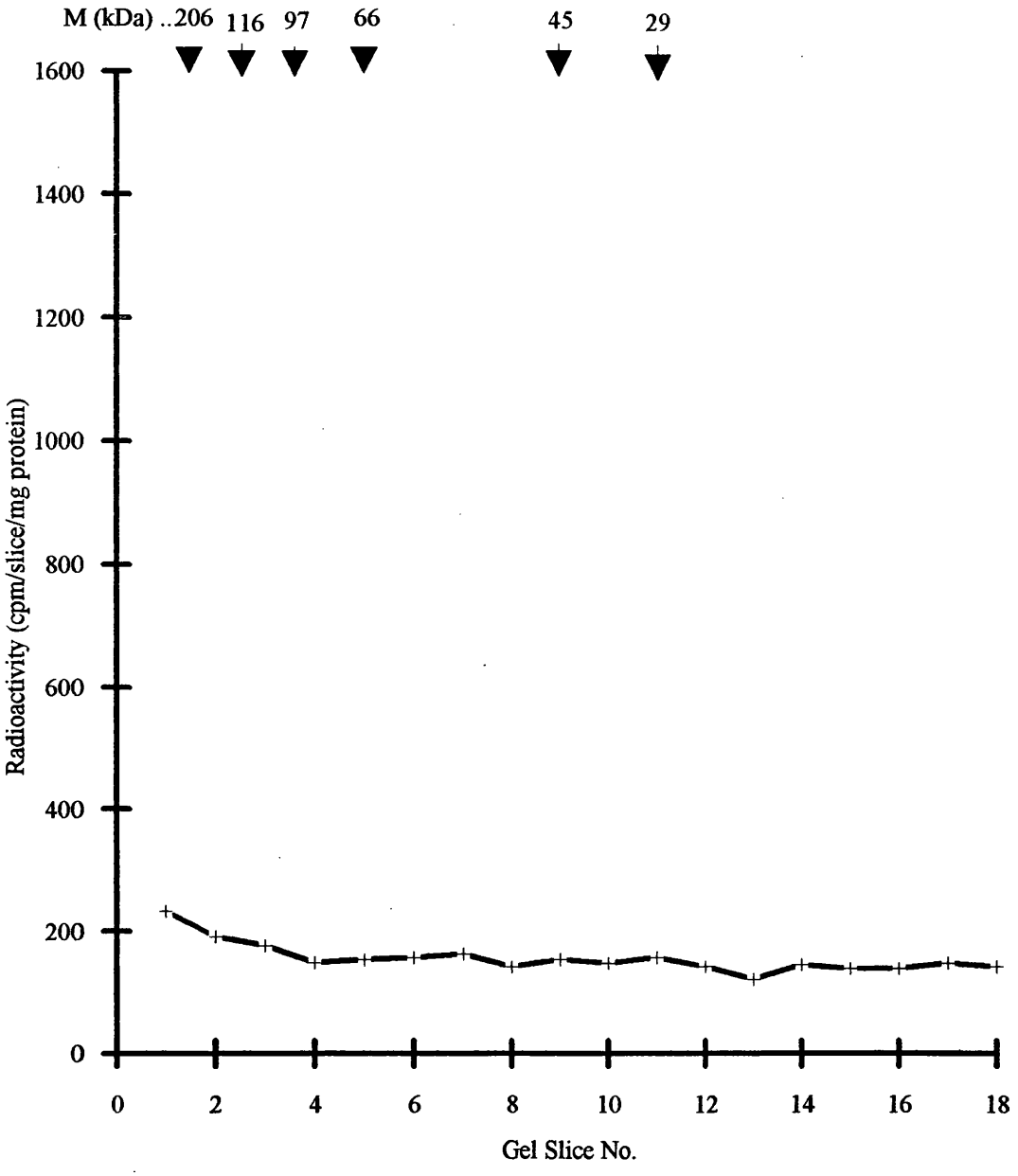


Figure 5.6 Cytochalasin B labelling of membranes of Sf9 cells infected with recombinant baculovirus N1. 48 hpi membranes were incubated with cytochalasin B (2.2.3.7), solubilised, and immunoprecipitated using the anti N-terminal GLUT1 antiserum. Samples were incubated with cytochalasin B in the absence of glucose. No radioactive labelling peak is detected on SDS-PAGE on a 12% acrylamide gel.

sequence analysis and the use of specific anti- peptide antibodies showed that this fragment represents most of the C-terminal half of the molecule. More specifically, the use of a range of proteolytic and chemical fragmentation techniques resulted in the mapping of the cytochalasin binding site to the cytoplasmic loop area between transmembrane segments 10 and 11. Clark and Holman (1990) demonstrated that the 18 kDa tryptic fragment could be labelled with cytochalasin B after tryptic digestion, demonstrating that not only does the cytochalasin B binding site exist on this fragment, but that its conformation following cleavage remains intact. This verifies the hypothesis that the cytochalasin B binding site is present on this half of the molecule. Closer identification of the binding area has located it between residues 347 and 456, probably between TM 9 and TM 10 (Cairns et al, 1987). Karim et al (1987) however, have stated that the internal ligand binding site is near to residue trp 388. These two statements are not necessarily contradictory to each other, since the three dimensional structure of the protein may be such that both are fully accurate.

The independent expression of the C-terminal putative six transmembrane helixes and associated non- membrane regions would potentially be important support for the localisation of the internal glucose binding site as being within the C-terminal half. Moreover, it would be an important test for the hypotheses of Karim et al (1987) that the N-terminal half is probably not required for the binding of ligands; and Mueckler et al (1985) that both glucose binding sites and the transmembrane transport channel exist between TM 7 and TM 10, and that the C-terminal half of the molecule could therefore function alone. Cytochalasin B labelling of this fragment would also further identify it as glucose transport protein, beyond the evidence of DNA restriction analysis and Western blotting.

However, figure 5.7, as representative of many similar experiments, clearly demonstrates that the independently expressed C-terminal half of the

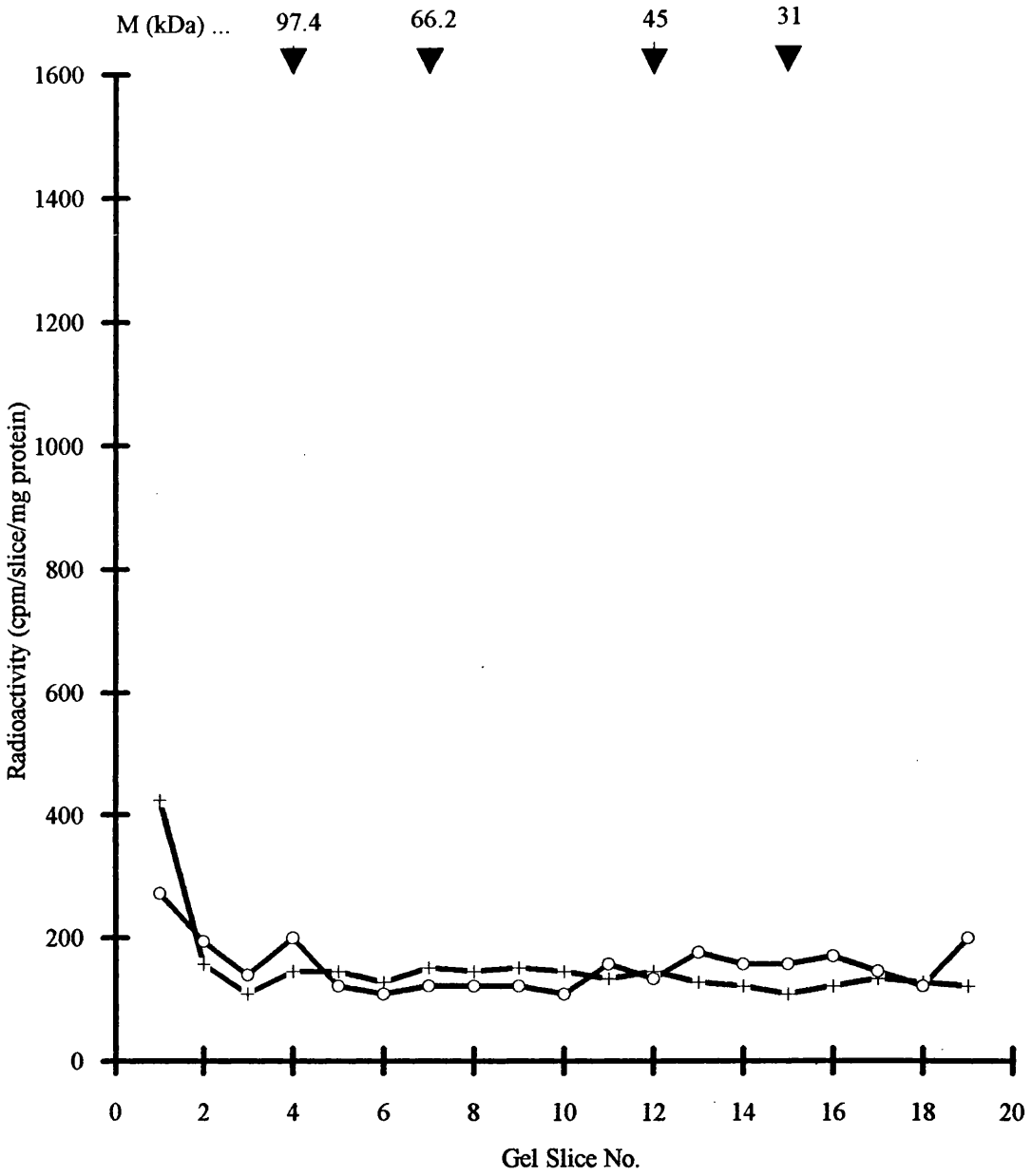


Figure 5.7 Cytochalasin B labelling of the C-terminal GLUT1 half encoded by the R12 recombinant baculovirus, as expressed in Sf9 cell membranes. Membranes were photolabelled and immunoprecipitated using the anti-C-terminal GLUT1 antibody (2.2.3.6 and 2.2.3.7), in the presence (o) and absence (+) of 200mM D-glucose. No labelling is detected following SDS-PAGE on a 12% acrylamide gel.

GLUT 1 glucose transport protein is not detectably labelled by [^{3}H]-cytochalasin B. In many cases when carrying out the labelling experiments on the cells expressing R12, the lowest molecular weight standard (lysosyme) at 14.4 kDa could not be visualised (on a 12% polyacrylamide gel), possibly due to insufficient loading. Because of this, it could be supposed that a labelling peak at a molecular weight of 25 kDa may have migrated beyond the end of the gel. In order to demonstrate unequivocally that no labelling had occurred at lower molecular weights, the labelled and immunoprecipitated samples were subjected to electrophoresis on a 15% polyacrylamide gel, including increased loading of molecular weight standards. These results, shown in figure 5.8, clearly demonstrate that, when expressed in the absence of the N-terminal six transmembrane regions of the protein, the C-terminal portion does not appear to posses a functional cytoplasmic-side glucose binding site. In insect cells at least, the C-terminal half of GLUT 1 can apparently not function alone.

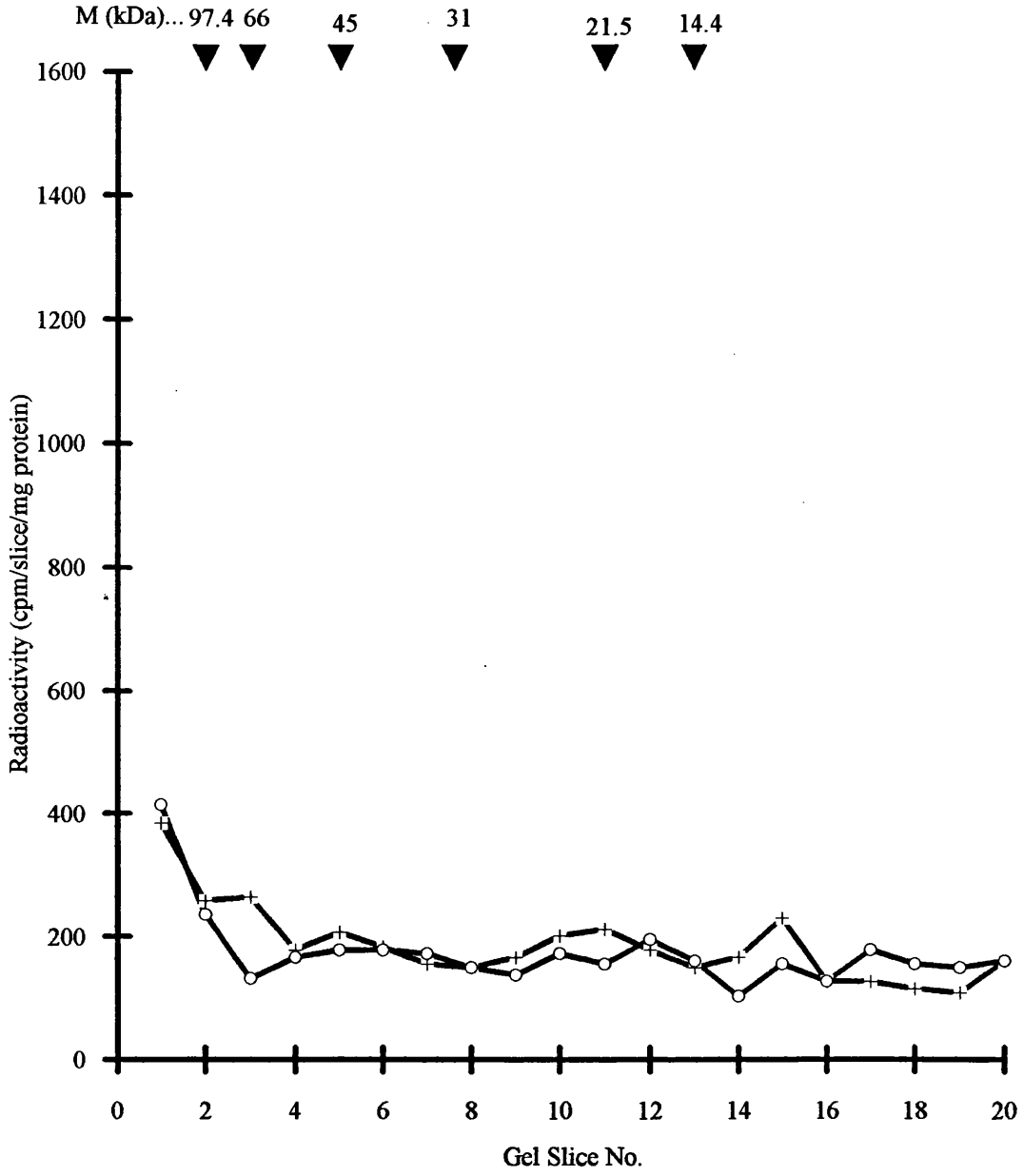


Figure 5.8 15% polyacrylamide gel analysis of immunoprecipitated cytochalasin B labelled membrane samples of Sf9 cells infected with the recombinant baculovirus R12, in the presence (o) and absence (+) of 200mM D-glucose.

5.2 ATB-BMPA LABELLING

5.2.1 INTRODUCTION

In 1982 Holman and Rees described the use of glucose analogues as inhibitors of sugar transport from the external surface of adipocytes. The use of side- specific agents for the inhibition of the glucose transporters was very important in the determination of the mode of action for transport. The development of a specific photolabel for the external site of erythrocyte glucose transporters was therefore important (Holman and Rees, 1987). ASA-BMPA is an azidosalicoyl derivative of bis (D-mannose). Its use resulted in important support for the conformational change hypothesis of glucose transport (by demonstrating that cytochalasin B and ASA-BMPA could not simultaneously occupy the same molecule, therefore suggesting that only one binding site is available at a time, per molecule). As a photolabel, it was also possible, by the use of enzymic fractionation, to determine the site of binding. Holman and Rees, 1987, placed the external binding site to be near to residue tryptophan 363. ATB-BMPA, another bis-mannose derivative (2-N-4-(1-azi-2,2,2-trifluoroethyl)-benzoyl-1,3- bis(D-mannos-4-yloxyl)-2-propylamine), has been found to label the transporter more efficiently than ASA-BMPA (Holman, 1989). The structure of ATB-BMPA is illustrated in figure 5.9 (from Clark and Holman, 1990)

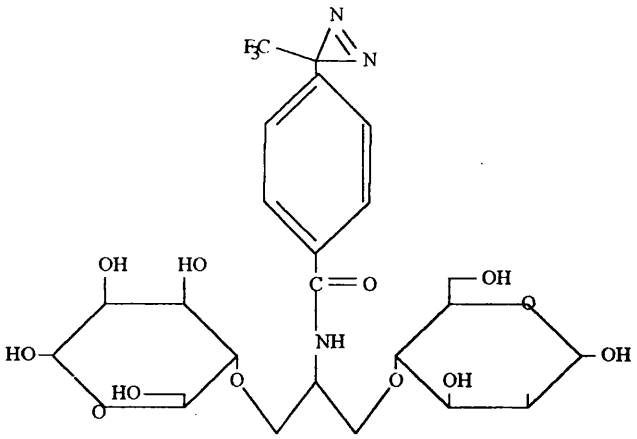


Figure 5.9 Structure of ATB-BMPA.

In the same way that a cytochalasin B labelling experiment works, the ATB-BMPA labelling profile of RBC is a broad peak between 55 and 65 kDa molecular weight, corresponding to the molecular size of the transporter. As with cytochalasin B, the labelling is almost completely displaceable by D-glucose. ATB-BMPA labelling of human erythrocytes is shown in figure 5.10. The labelling peak corresponds to the molecular weight of the GLUT 1 transporter, at between about 50 and 65 kDa.

5.2.2 ATB-BMPA LABELLING EXPERIMENTS ON EXPRESSED FULL-LENGTH PROTEIN

Uninfected Sf9 cells, and cells infected with the "wildtype" baculovirus were tested for ATB-BMPA labelling, to determine whether the cells' native hexose transporter(s) would be labelled by this ligand, and immunoprecipitated with the anti-GLUT 1 antiserum. Since no cytochalasin B labelled species was immunoprecipitated with the anti- GLUT 1 antibody, no immunoprecipitated labelled species was expected to be detected using ATB-BMPA. Neither were found to bind this ligand and be immunoprecipitated.

If the full-length transporter expressed in Sf9 cells had a functional external glucose binding site the ATB-BMPA labelling experiment would be

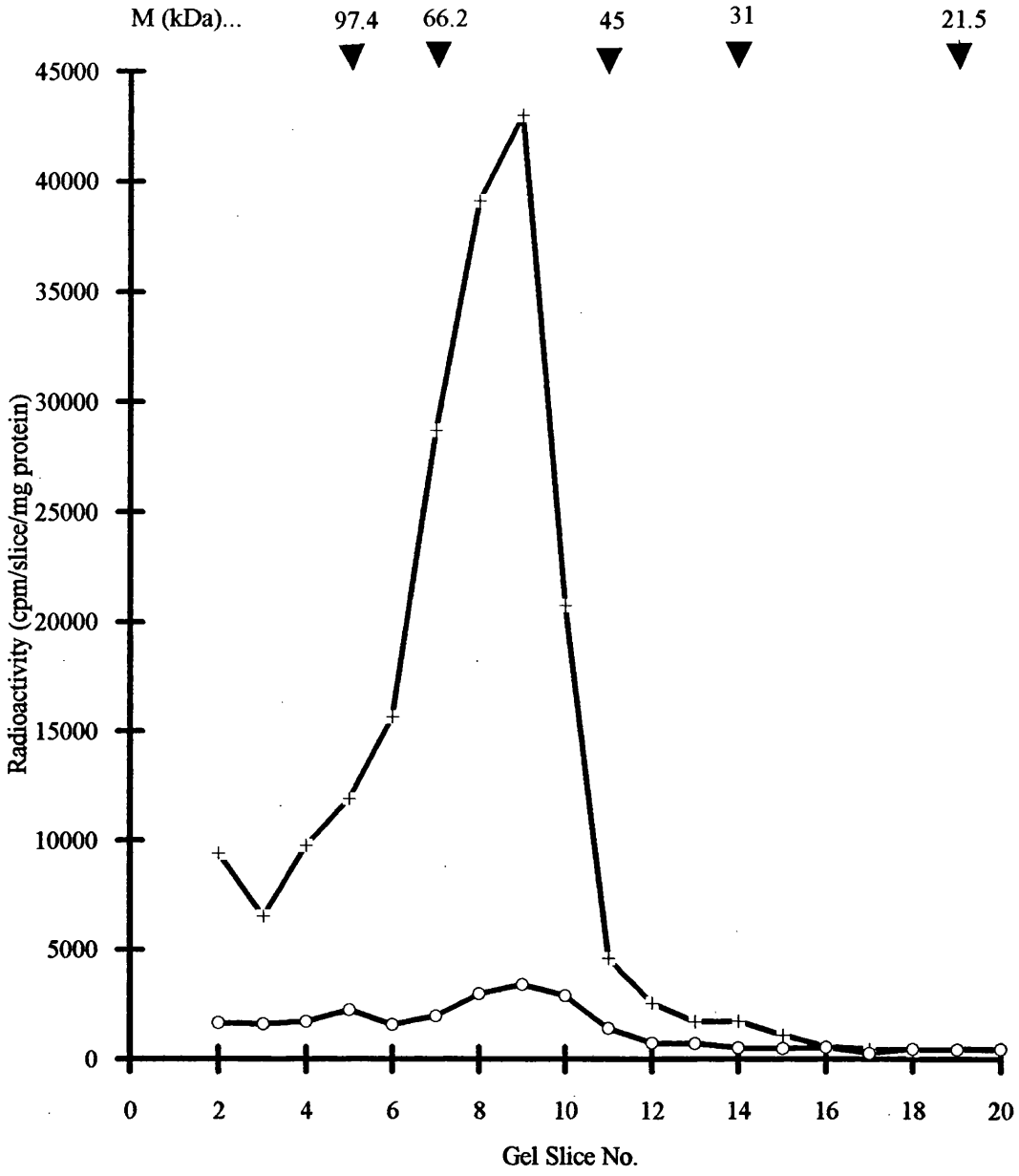


Figure 5.10. ATB-BMPA labelling of erythrocyte membranes. Membranes were incubated with ATB-BMPA with (o) and without glucose (+), and irradiated. Solubilised samples were immunoprecipitated using the anti -C-terminal GLUT1 antibody, and subjected to SDS-PAGE on a 10% acrylamide gel. Vertical slices were made of the gel lanes, and the radioactivity (^3H) content of each slice measured. The labelling peak corresponds to a molecular weight of between 55 and 65 kDa.

expected to show a labelling peak at ~50 kDa, the size of the expressed transporter.

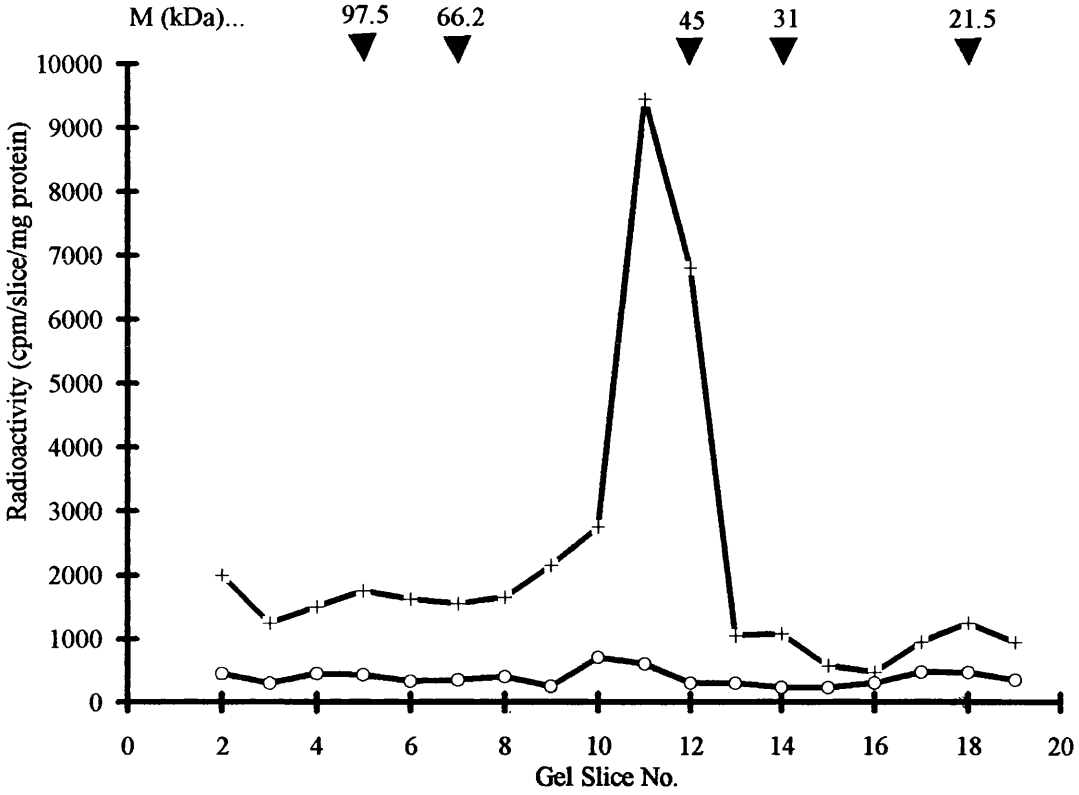
Figure 5.11 shows that ATB-BMPA labels the E4.2 full length transporter protein with a clear peak at around 50 kDa. This peak is fully displaced by the presence of 200mM D-glucose. ~50 kDa corresponds to the observed molecular weight of the E4.2 GLUT1 transporter protein as demonstrated by Western blotting using the anti-GLUT1- C-terminal antibody.

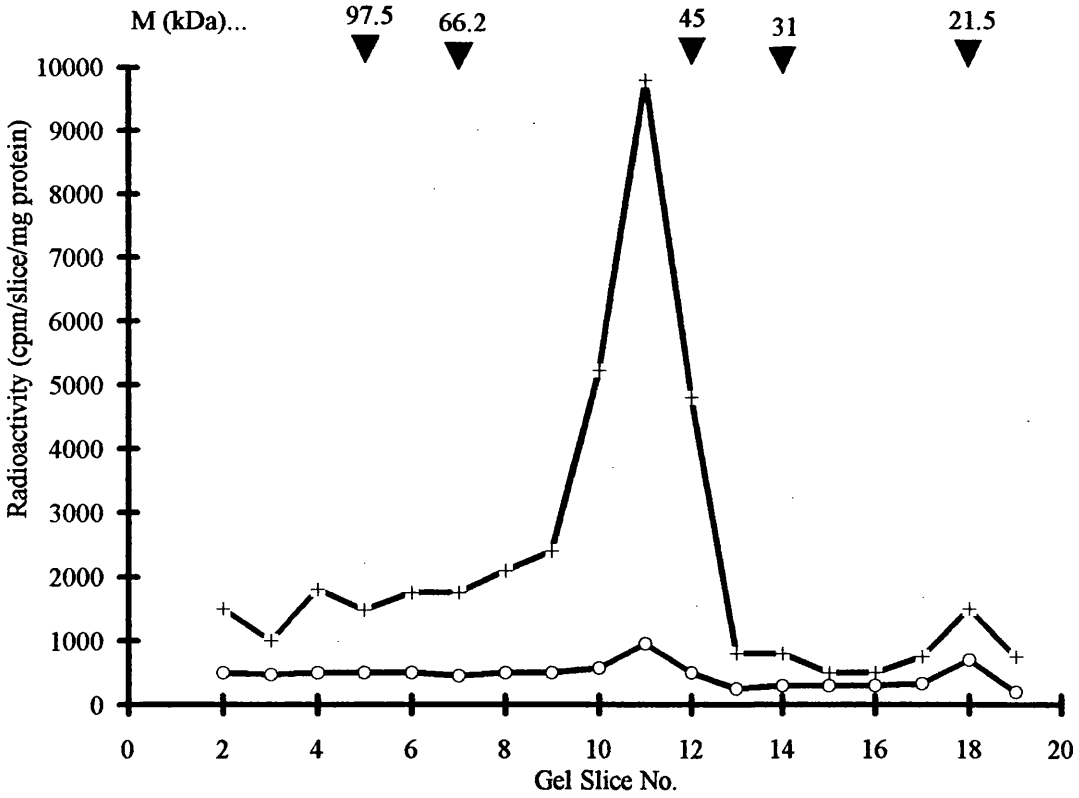
In order to determine whether the E4.2/Sf9 expressed GLUT1 molecules were expressed largely at the surface of the cell, or whether a significant proportion of them were intracellularly localised, cells were incubated with 0.025% digitonin for eight minutes prior to a normal labelling experiment. The results of this are illustrated in figure 5.11b, as compared to figure 5.11a. This appears to indicate that either a great majority of the E4.2 GLUT1 molecules are expressed on the surface of the cell, or that any intracellular E4.2 GLUT1 molecule are not in an appropriate conformation for the binding of the ATB-BMPA ligand.

As was found with cytochalasin B labelling, when the labelling profiles of GLUT1 in RBC membranes and the insect cell membrane expressed E4.2 GLUT1 (labelling concomitantly performed) are compared (figure 5.12), two principal differences are observed. Firstly, there is the expected difference in the molecular weight at which the ligand labels. The transporter expressed in the insect cells is apparently smaller than that of RBCs, and corresponds to the size difference observed by Western blotting. However, using roughly matched samples (as in the cytochalasin B experiments, the samples were approximately matched by visual assessment of immunogenic protein content at the appropriate weight, from Western blots), the RBC sample shows much more labelling with ATB-BMPA than the E4.2 sample. From this experiment, it is not possible to determine whether this difference is due to a reduced binding affinity when the protein is expressed in insect cells, or whether a substantial

Figure 5.11 **A.** ATB-BMPA labelling of Sf9 cells infected with recombinant baculovirus E4.2 (cells taken at 48 hpi). The cells were incubated with ATB-BMPA, and irradiated for 45 seconds (as described in section 2.2.3.8). The cells were solubilised, immunoprecipitated for two hours using the anti- C-terminal GLUT1 antibody, and subjected to SDS-PAGE on a 12% acrylamide gel. The radioactivity of the gel slices made was measured. Incubations were made in the presence (o), and absence (+) of 200mM D-glucose. This clearly shows that the ~50 kDa binding peak is displaced by D-glucose.

B. ATB-BMPA labelling of digitonin permeabilised Sf9 cells infected with recombinant baculovirus E4.2. Cells were incubated with 0.025% digitonin for eight minutes before incubation with the photolabel. The digitonin was not removed before the labelling. The cells were solubilised, immunoprecipitated for two hours using the anti- C-terminal GLUT1 antibody, and subjected to SDS-PAGE on a 12% acrylamide gel. The radioactivity of the gel slices made was measured. Incubations were made in the presence (o), and absence (+) of 200mM D-glucose. There is clearly no appreciable difference in the height of the labelling peak between whole cells and permeabilised cells.


M (kDa)...
97.5
66.2
45
31
21.5
Radioactivity (cpm/slice/mg protein)
Gel Slice No.


M (kDa)...
97.5
66.2
45
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Radioactivity (cpm/slice/mg protein)
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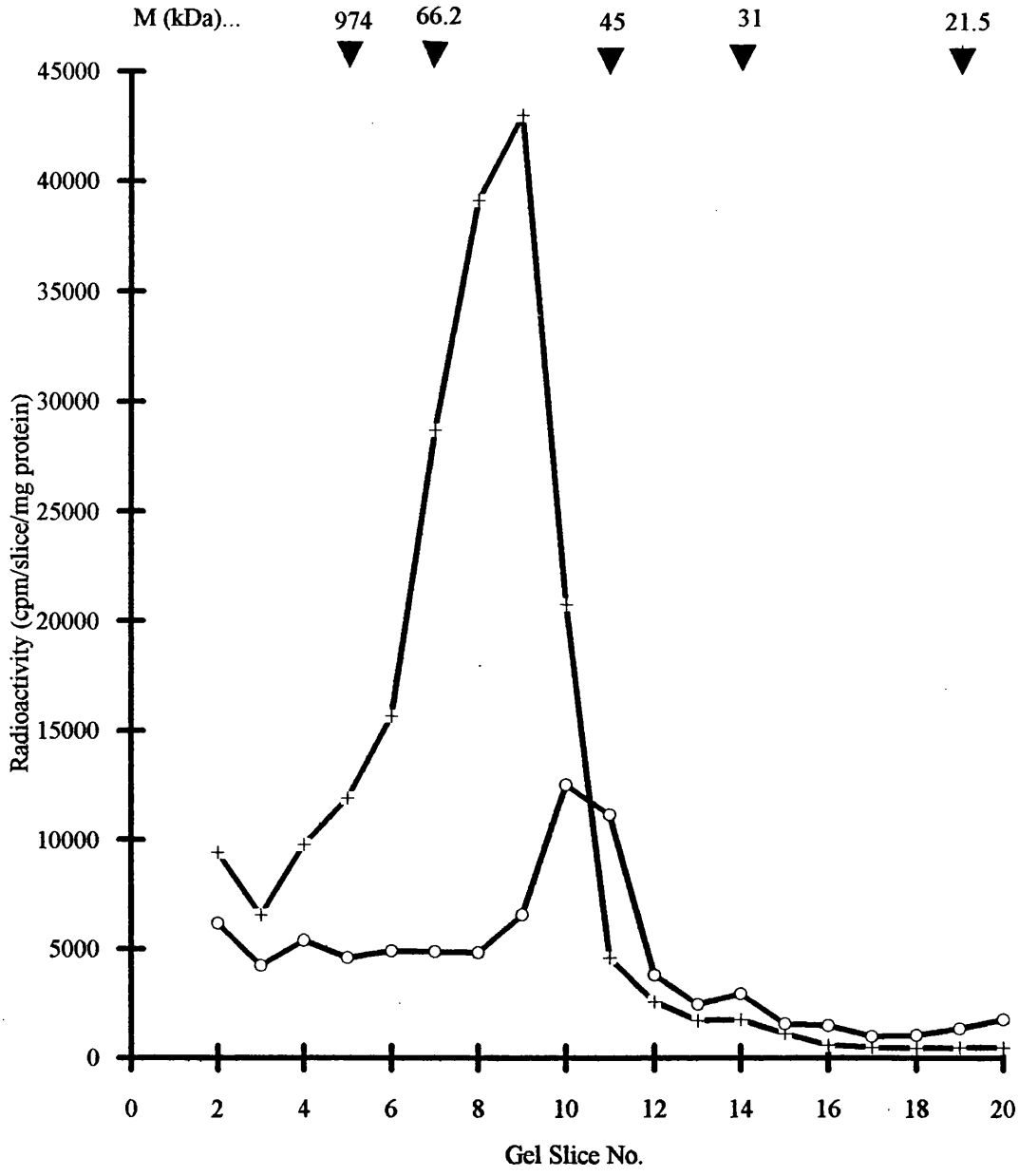

Figure 5.12. Comparison of anti C-terminal GLUT1 antiserum immunoprecipitates of ATB-BMPA labelling of erythrocyte membranes (+) and baculovirus E4.2 infected Sf9 cell membranes (o). Sf9 cells and RBC membranes were photolabelled, immunoprecipitated (using the anti C-terminal GLUT1 antibody) and subjected to SDS-PAGE on a 12% acrylamide gel (as described in chapter 2) simultaneously. This figure illustrates the different mobilities of the photolabelled species of RBC and E4.2 infected Sf9 cells.

number of the expressed proteins are detectable by the anti GLUT1 antisera, but do not have functionally appropriate external glucose binding sites.

5.2.3 ATB-BMPA LABELLING EXPERIMENTS ON N-TERMINAL HALF PROTEIN

Since it is generally accepted that neither of the glucose transporter's binding sites are located anywhere on the N-terminal half of the molecule, no labelling of cells infected with the N-terminal GLUT1 expressing N1 baculovirus was expected with this ligand.

Figure 5.13 demonstrates the consistent finding that on the usual exposure to ATB-BMPA, and following immunoprecipitation with the anti N-terminal anti GLUT1 antiserum, no labelling at any molecular weight could be detected in cells expressing the N1 protein. Figure 5.14 is presented as confirmation that the anti N-terminal antiserum successfully immunoprecipitates the ATB-BMPA labelled E4.2 transporter protein.

5.2.4. ATB-BMPA LABELLING EXPERIMENTS ON C-TERMINAL HALF PROTEIN

The labelling studies of Clark and Holman (1990) of tryptic digests of the erythrocyte glucose transporter demonstrated that cytochalasin B bound to the 18 kDa fragment which corresponds to a large portion of the C-terminal half of the molecule (but excluding the cytoplasmic C-terminal tail, and without most of the putative central cytoplasmic loop). Cytochalasin B was found to bind before digestion, to be detected to be associated with this fragment. It would also bind to this fragment following digestion. However, if the glucose transporter was labelled with ATB-BMPA before trypsin treatment, the digestion was prevented. This was considered to be indicative of conformational change, that when the transporter is in the outward- facing conformation (i.e. binding an external- site specific ligand) the trypsin digest

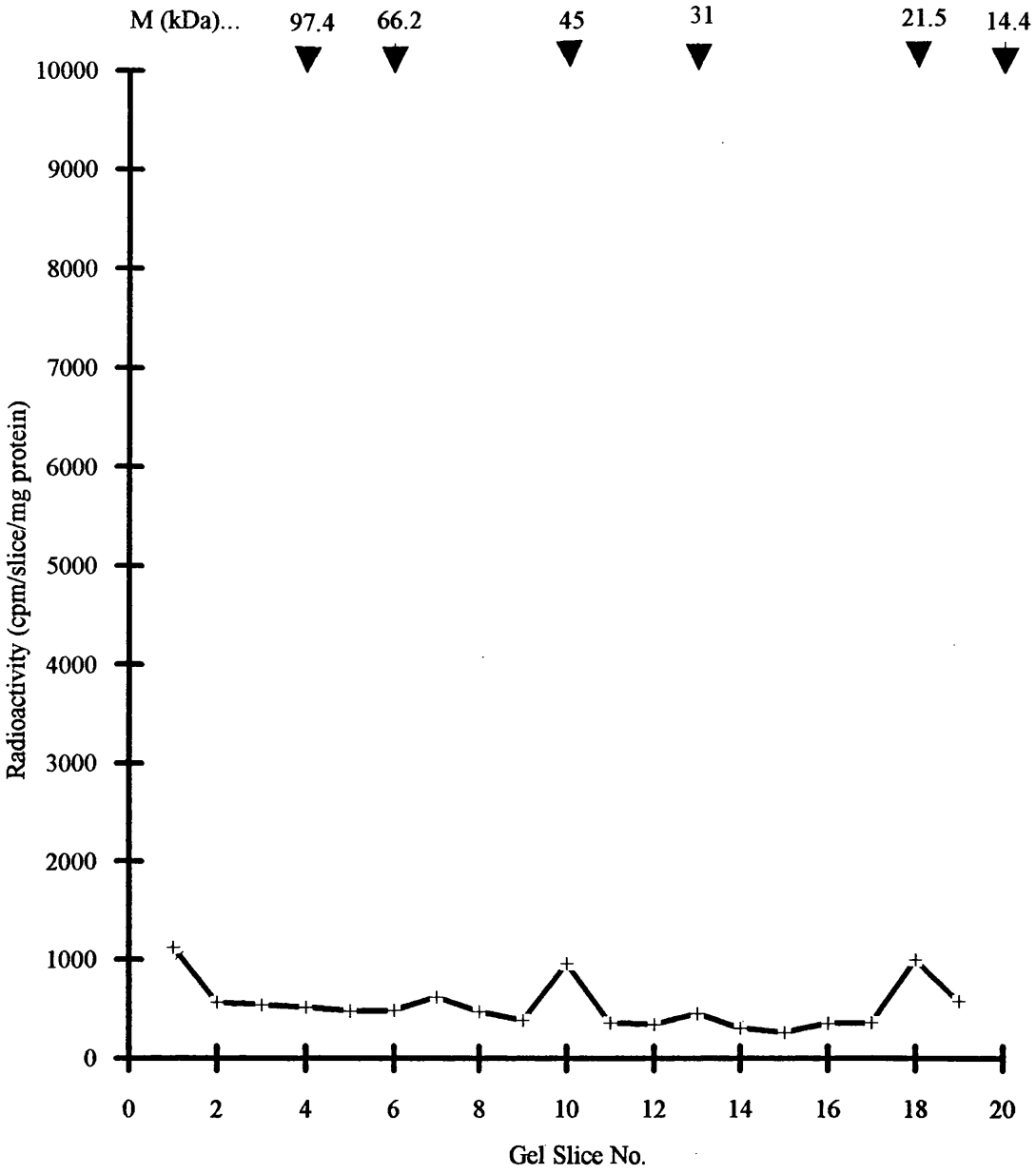

Figure 5.13 ATB-BMPA labelling of Sf9 cells infected with recombinant baculovirus N1. Labelled samples (section 2.2.3.8) were immunoprecipitated (2.2.3.6) using the anti N-terminal half GLUT1 antiserum, and subjected to SDS-PAGE on a 12% polyacrylamide gel. Samples were incubated in the absence of glucose. The radioactivity content of the gel slices made was measured. No distinct labelling species is detectable.

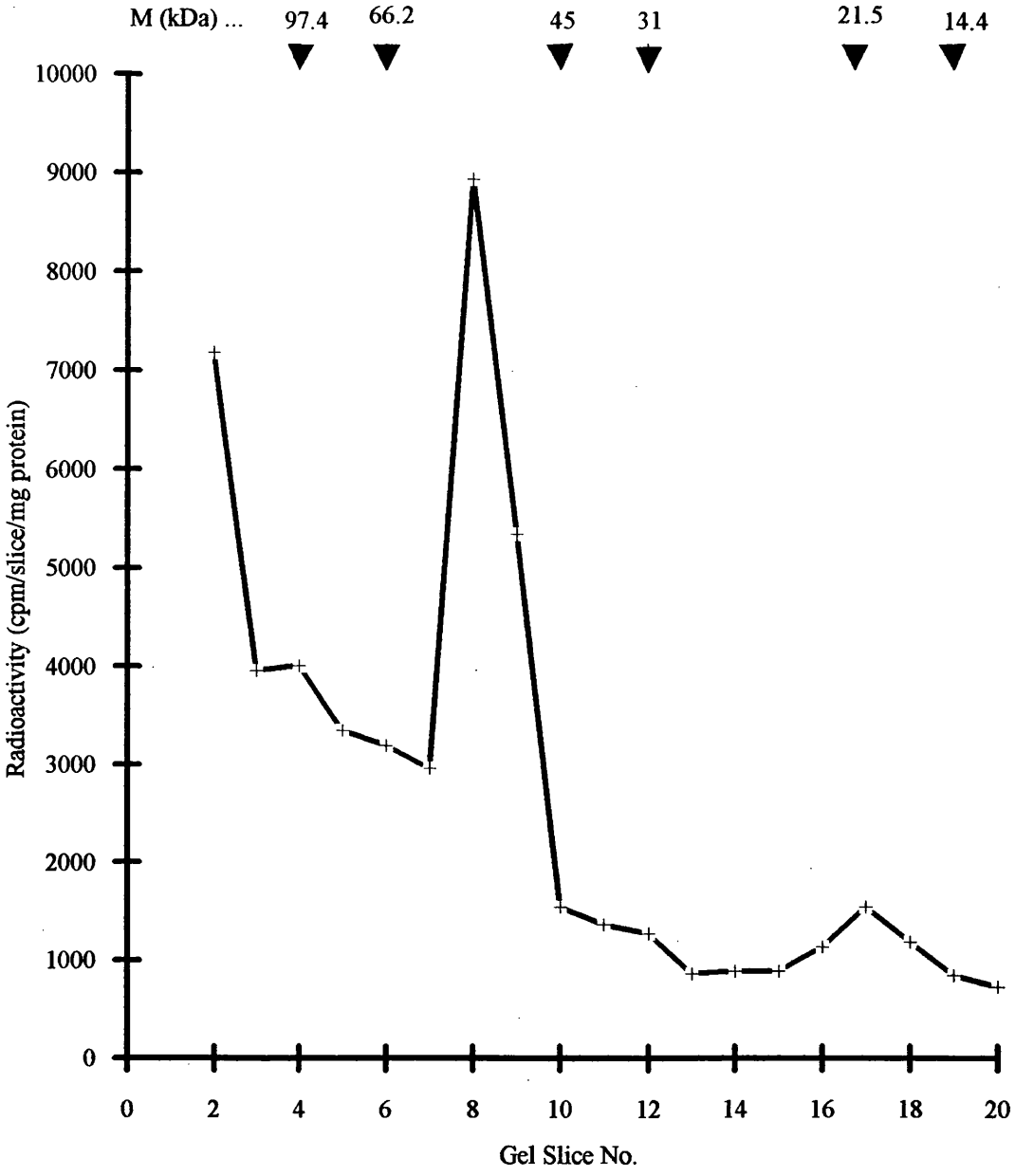

Figure 5.14 ATB-BMPA labelling of Sf9 cells expressing the E4.2-encoded GLUT1 protein (cells taken at 48 hpi). The cells were incubated with ATB-BMPA, and irradiated for 45 seconds (as described in section 2.2.3.8). The cells were solubilised, and immunoprecipitated for two hours using the anti- N-terminal half GLUT1 antibody, before being subjected to SDS-PAGE on a 12% acrylamide gel. This demonstrates immunoprecipitation of the E4.2/Sf9 ATB-BMPA-labelled species by the anti N-terminal GLUT1 antibody.

site was not available to the trypsin molecule. On pre- digestion with trypsin, ATB-BMPA could not be shown to bind. This was considered to be an indication that the 18 kDa C-terminal fragment was perhaps locked into an internal- facing conformation, allowing only the binding of ligands specific to the internal binding site. However, the external binding site has been shown to exist on the C-terminal half of the molecule (Karim et al, 1987; Hashirimoto et al, 1992).

Despite the observations by Clark and Holman (1990) that the 18 kDa C-terminal fragment was apparently locked into an inward- facing conformation, the C-terminal half expressed in the Sf 9 cells is a quite different, and larger polypeptide. Since the external binding site is considered to exist on this half of the molecule it was considered that labelling might be observed. Based on the observation of apparent "dimer" structures as seen by approximately 50 kDa anti-GLUT 1 antibody immunoreactive bands on Western blotting (see chapters 3 and 4), it was considered that if an association of the C-terminal polypeptides is the case, than perhaps this may have some bearing on either the structural or functional status of the molecules. That is, labelling was anticipated to be detected at either a molecular weight of around 25 kDa, or at the weight of the higher molecular weight species observed on Western blotting.

The failure of the ligand to bind at any molecular weight of protein expressed by R12 baculovirus infected cells was consistently shown, and is demonstrated in figure 5.15. This shows that the C-terminal half of the protein, when expressed alone in this way, does not possess a functional external glucose binding site. However, since the C-terminal half was also demonstrated to fail to bind cytochalasin B, this could not support the prediction that a C-terminal half molecule may be locked into an inward- facing conformation.

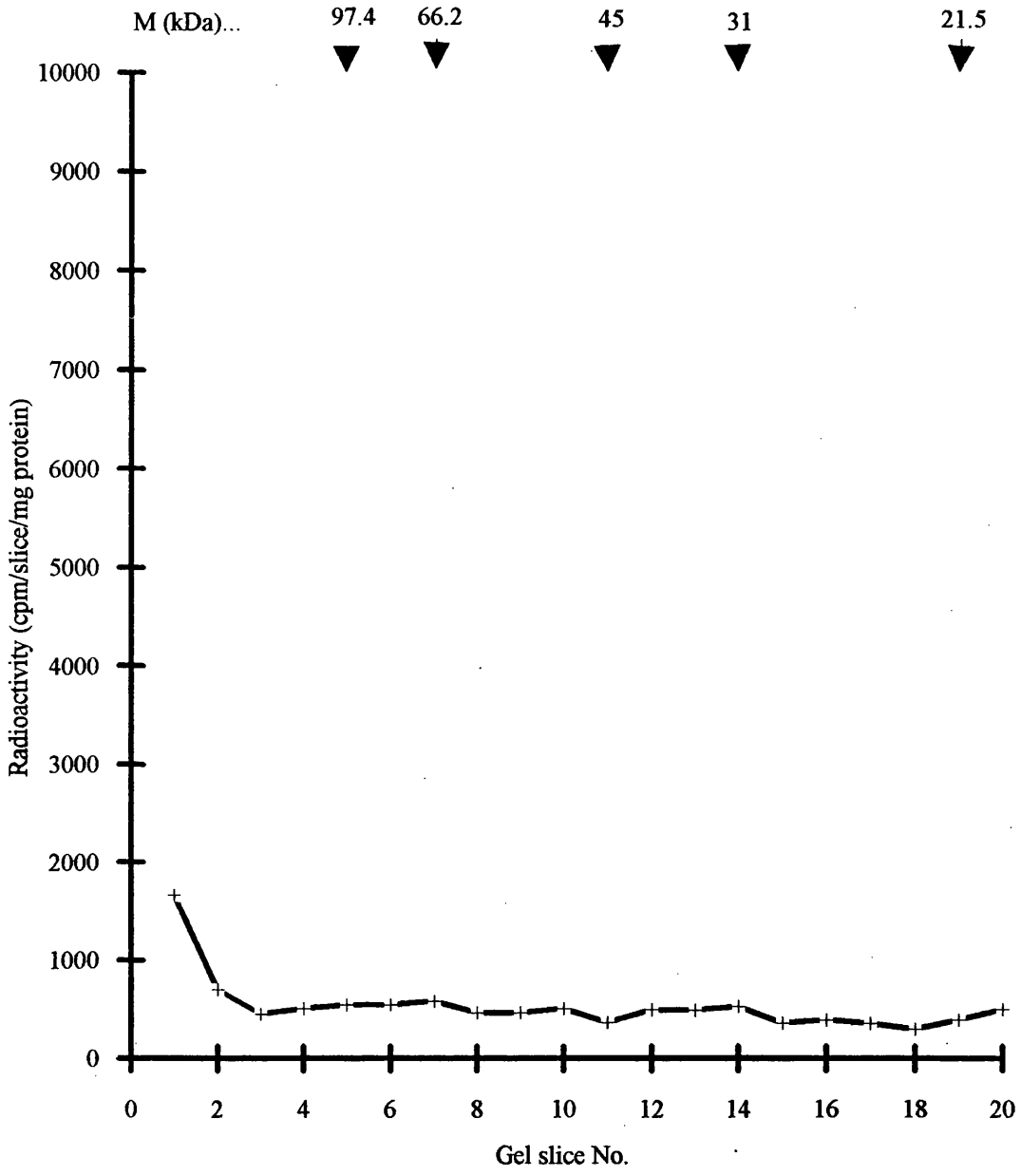

Figure 5.15 ATB-BMPA labelling of Sf9 cells infected with recombinant baculovirus R12. Cells were incubated with ATB-BMPA, immunoprecipitated using the anti C-terminal GLUT1 antibody, and subjected to SDS-PAGE on a 12 % acrylamide gel, according to sections 2.2.3.2, 2.2.3.6 and 2.2.3.8 of chapter two. No anti-GLUT1 antibody reactive ATB-BMPA-labelled peak was detected. These data are representative of an experiment repeated numerous times.

5.3 TRANSPORT MEASUREMENT

Glucose transport measurements of cells infected with recombinant baculovirus E4.2 (expressing the full-length GLUT1 protein) were exhaustively tried, by way of uptake assays of tritiated 2-deoxy-D-glucose. The basic method used is described in section 2.2.3.10 of Materials and Methods. It had been previously found that the endogenous sugar transporter of the Sf9 cells was capable of 2-deoxy-D-glucose transport, but that this transport could be inhibited by the presence of 400 mM fructose (A.E. Clark; unpublished).

Since GLUT1 ligand binding and glucose transport are not inhibited by fructose, fructose was added to all of the experimental conditions in order to minimise any background transport activity occurring by the insect cells' own transporters. As is clear from figure 5.16, the measurement of non-fructose inhibitable 2-deoxy-D-glucose uptake was not possible in cells infected with baculovirus. The findings were consistent in that there was no noticeable difference in the results of transport assays carried out on cells infected with AcMNPV-lacZ (wild type) virus and cells infected with either E4.2 (whole GLUT 1 transporter) or R12 (C-terminal half molecule). Despite fructose inhibition, some degree of 2-deoxy-D-glucose uptake was measurable in "mock" infected Sf9 cells (see fig.5.16). This is lost when cells are infected with baculovirus. On a number of occasions (without consistent reproducibility), uptake was apparently measured in cells infected with virus E4.2. However, the levels apparently measured were not higher than those measured for "mock" infected Sf9 cells . Since this was not reproducible, the occasional apparent transport measurement may therefore be the result of an inadequate infection.

In one transport assay, both the fructose inhibitable glucose transport, and the non-inhibited glucose transport levels measured (calculated per 10^6 cells) were seen to be much higher in cells infected with baculovirus E4.2, than

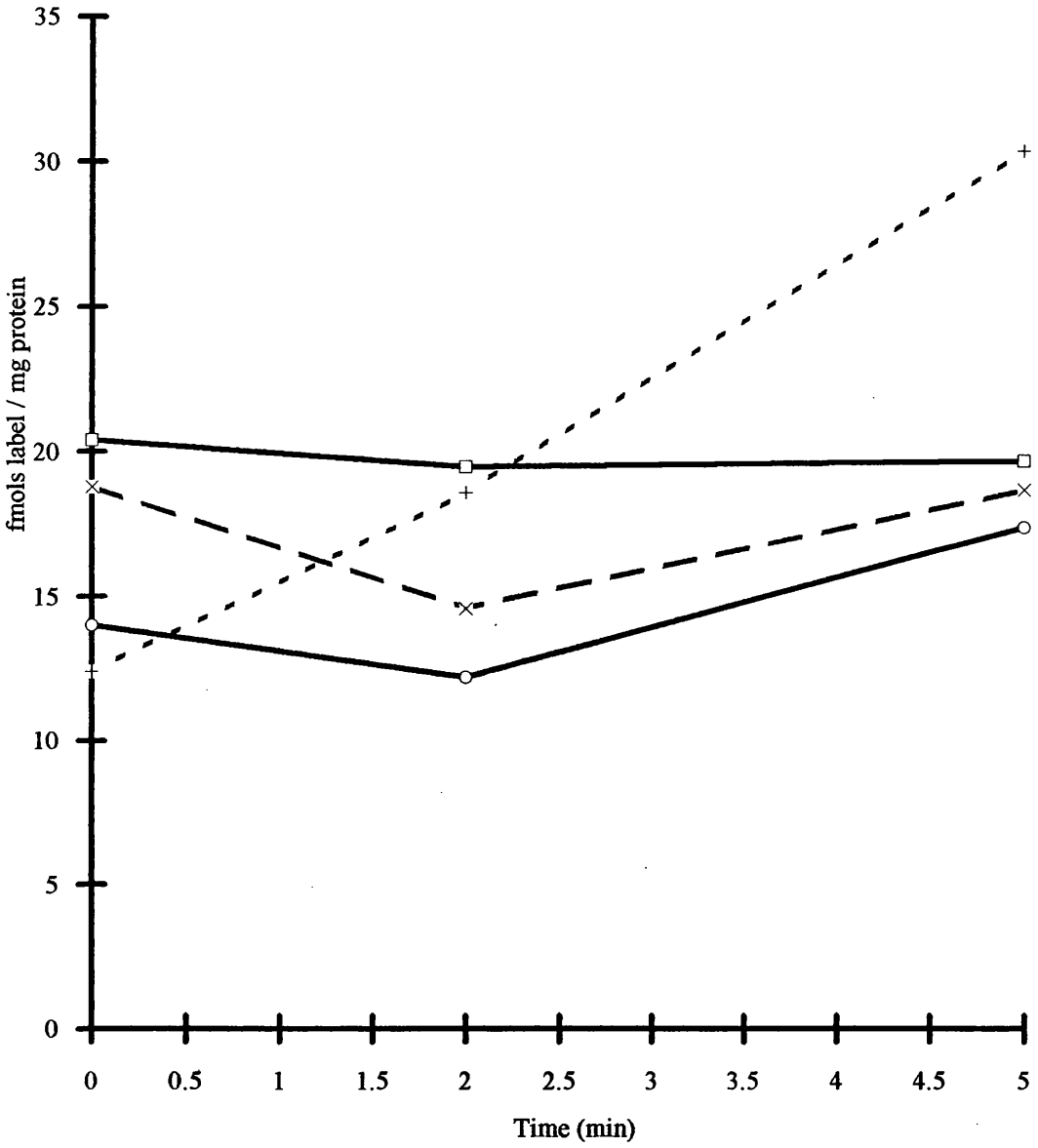

Figure 5.16 2-deoxy-D-glucose transport assay (see section 2.2.3.10) of Sf9 cells infected/mock infected, and assayed at 48 hpi. Cells were mock infected (+), infected with AcMNPV:LacZ (o), or infected with either recombinant baculovirus E4.2 (□), or R12 (x). All assay conditions included 400mM fructose.

in either cells infected with the wildtype baculovirus, or mock infected cells (see figures given in table 5.1). All figures shown in table 5.1 represent transport of 2-deoxy-D-glucose at two minutes transport, having taken away the figures measured for cells incubated with both fructose and glucose during the assay.

Infection of cells	fructose-inhibitable D-glucose transport (fmol/10^6cells)	non-inhibited D-glucose transport (fmol/10^6cells)	fructose inhibitable transport as a % of total.
mock	0.4	2.8	14%
"LZ"	0.2	1.9	10%
E4.2	0.8	5.7	14%

Table 5.1 2-deoxy-D-glucose transport in infected and uninfected Sf9 cells.

As is clear from table 5, although the transport measurements were apparently higher for the E4.2 infected cells, the reduction in transport measured by the addition of fructose, as a proportion, was not different between the two conditions. That is, although the measurements were higher, the increased transport measured was not due to the actions of a non-fructose inhibitable system. Although this result is interesting, in that it appears to demonstrate that the GLUT1 encoded by the E4.2 virus is not functional, the result was never reproduced, and the result shown in figure 5.16 is more typical of the results of glucose transport assays on these cells.

It was therefore considered to be highly likely that the measurement of hexose uptake was to be impossible. At 48 hpi, when expression of the GLUT1 protein was good, the cells were already in a state of considerable deterioration, as a consequence of the viral infection. It was considered that the damage sustained by the majority of the cells by this stage in the infection was such that the cells had become "leaky" as their membrane integrity was becoming compromised. The measurement of hexose transport at an earlier point of the viral infection would mean the measurement from very many fewer transporters (for example, the quantity of GLUT1-antibody immunoreactive material at 24 hours is less than four-fold lower than at 48 hours (by visual assessment of Western blots; see the E4.2 time course, chapter 4 section 4.1.2; and based on the relative quantification carried out on the R12 protein, which showed a very similar expression pattern).

5.4 SUMMARY

The results of this chapter have demonstrated that the GLUT 1 protein expressed in insect cells is successfully labelled by the ligands cytochalasin B and ATB-BMPA. The labelling in both cases is glucose displaceable, which demonstrates the presence and ability of both glucose binding sites to bind glucose. This indicates that the insect cell-expressed GLUT 1 glucose transporter has functional external glucose binding sites and functional internal glucose binding sites, when expressed as a full-length protein. However, the results also indicate that the labelling of both ligands appears to be significantly lower than labelling of the GLUT 1 protein in the native erythrocyte environment. This is considered to be either due to a reduced affinity in the protein when expressed in insect cells; or possibly that only a proportion of the GLUT 1 molecules bind the ligands. This finding is consistent with that from similar experiments carried out by Yi et al (1992), who also expressed GLUT 1 (human) in Sf 9 cells, using the baculovirus expression system. Although their expressed protein showed apparent differences to the Sf 9/ baculovirus expressed GLUT 1 protein described here when analysed by Western blotting (discussed in chapter 7), their protein was also found to bind cytochalasin B. Their findings, as those described here, were that the apparent concentration of active cytochalasin B binding sites was much lower than the quantity of immunoreactive protein present. No other study has to date provided labelling or binding data for external binding site ligands for glucose transport proteins expressed in this way.

These results apparently indicate that the functional (i.e. will bind the ligand ATB-BMPA) E4.2/Sf9 expressed GLUT1 molecules are largely present on the surface of the Sf9 cells. However, the results of the digitonin-permeabilisation experiments were not clearly reproducible, so this result is not

considered to be conclusive evidence that the functional molecules are largely cell-surface localised.

These results concluded that the functional status of the full-length GLUT1 glucose transporter by hexose transport could not be determined using this system. Yi et al (1992) also found that on their expression of GLUT1, no hexose transport measurements were possible. The assessment of functional status of their GLUT1 protein was determined by cytochalasin B binding. They also concluded that measurements could not be made because the cells had become "leaky" at the time of optimal (or even reasonable) glucose transporter expression.

Woon et al (1991)however, on their expression of GLUT4 protein, apparently found that measurement of hexose transport was possible. They found that the increase in GLUT 4 expression measured over 78 hours of infection, was accompanied by an increase in 2-deoxy-D-glucose uptake by the cells. This transport was found to be inhibitable by forskolin (as would be expected of GLUT 4), with an apparently different affinity than that of the endogenous transporters, for which they recorded a significant background rate of transport. Why transport measurements were demonstrated to be possible for the GLUT 4 (Woon et al) isoform but not for GLUT 1 (described here, and by Yi et al, 1992) is not known.

Results presented here have shown that, expressed independently of one another, neither of the two glucose transporter halves bind either of the ligands. This indicates that, despite possessing the secondary structural positions of both the external and the cytoplasmic side glucose binding sites, the C-terminal half of GLUT 1 expressed alone in this way does not have functional glucose binding sites. That is, the suggestion of Karim et al (1987) that the residual N-terminal half region of the molecule (as a tryptic digest in their work, and therefore still present in the membrane) is not required for ligand binding is not supported. The hypothesis made by Mueckler et al

(1985) that a fragment containing only the transmembrane regions TM 7 to TM 10, with associated links, would be fully functional, is shown here (for a larger fragment, expressed in a non- native environment) to be unsupported.

CHAPTER 6.

DUAL INFECTION

6.1 INTRODUCTION

One of the original aims of the project was to express the C-terminal half of GLUT 1, in insect cells, as though it were a protein in its own right. The hypothesis was that both glucose binding sites were resident on this part of the molecule. As has been described in chapters 2 and 5 , this is a hypothesis based on, and supported by, a broad body of evidence. As has been described in chapter 5 however, this hypothesis could not be supported by the work described in chapter 5.

However, on the basis of a number of factors, the aim of the project was later modified. The C-terminal half protein expressed independently of any N-terminal half protein or whole GLUT 1 protein, could not be shown to bind the ligands. Yet the body of evidence for the localisation of the binding sites appears to contradict this. It was concluded that the binding sites may indeed be present on this part of the molecule, but that part of the N-terminal half of the molecule must be necessary to permit the binding to occur. In a paper by Karim et al (1987), the authors speculatively conclude that the C-terminal half of the molecule could function alone, on the basis of proteinase digestion of, and ligand binding to the GLUT 1 protein. However, the authors also offer the alternative conclusion that proteolysis with trypsin only "nicks" the transporter, and that the two major fragments observed may actually be held together as a functional unit during ligand binding and labelling. Therefore, evidence in which the transporter is fragmented, but where no fragment is isolated, cannot rule out the possibility of N-terminal involvement in ligand binding.

It was consistently observed, on Western blotting experiments of the R12 C-terminal protein, that the 25 kDa expected - size protein was not the only species to be detected by the anti GLUT 1 antibody. Figure 6.1 illustrates the second major species to be detected. This species has an M_r of around 50

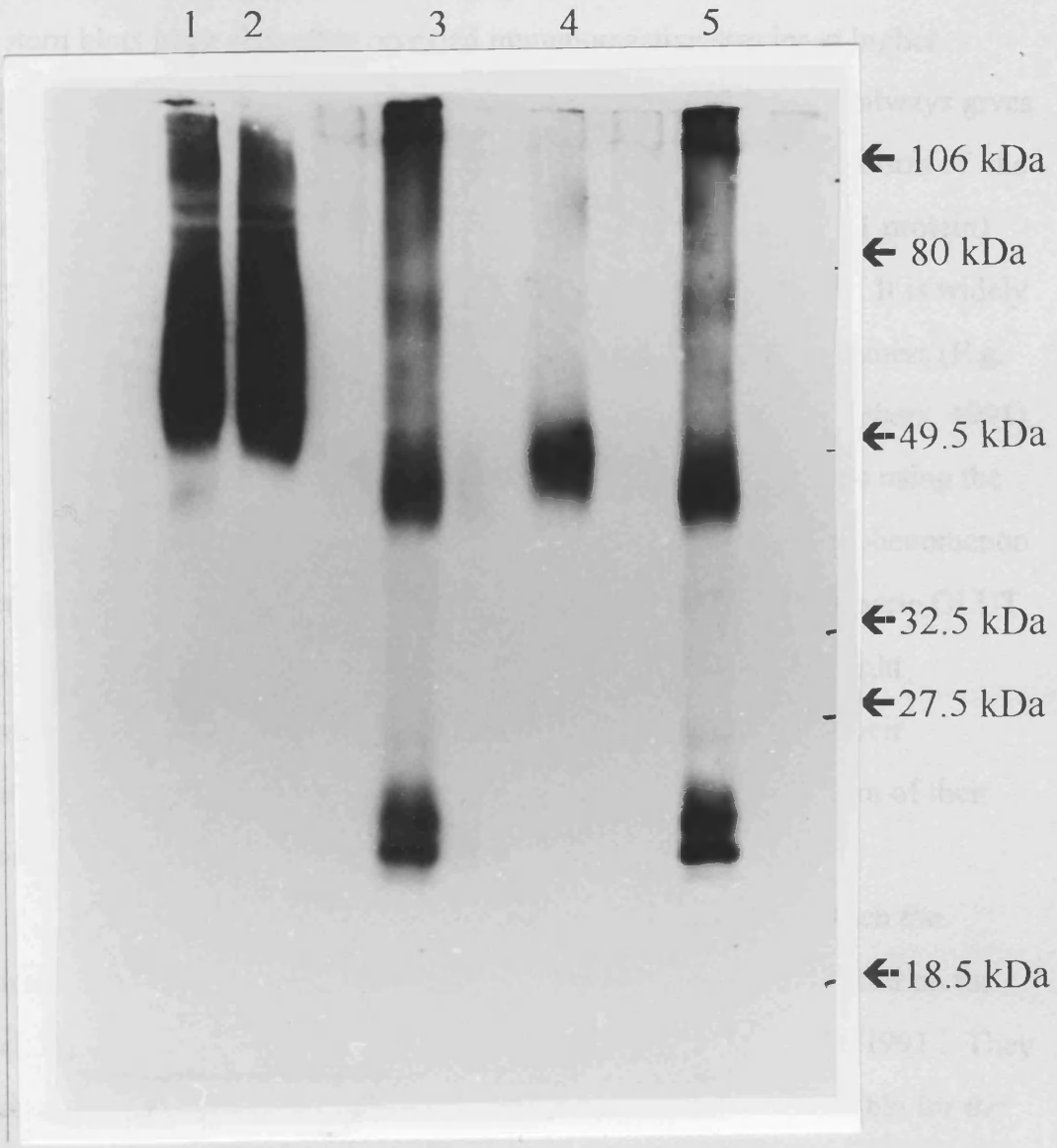

Figure 6.1 Western blot of a 12% SDS-PAGE, using anti C-terminal GLUT1 antibody. Lanes 1 and 2 show red blood cell membrane GLUT1 protein. Lanes 3 and 5 show anti-GLUT1 antibody immunoreactive material in Sf9 cell membranes, at 48 hpi, with recombinant baculovirus R12. Lane 4 shows immunoreactive material of Sf9 cell membranes similarly infected with recombinant baculovirus E4.2. Note the presence of an immunoreactive species of apparently ~50 kDa in R12 infected membranes (lanes 3 and 5) in addition to the C-terminal half ~25 kDa size protein.

kDa; approximately twice that of the C-terminal 25 kDa. As figure 6.1 shows, the mobility is slightly greater than the mobility of the E4.2 GLUT 1 protein. Western blots have also often revealed immunoreactive species at higher molecular weights, but the 50 kDa band is consistently shown and always gives a strong signal. It was concluded that this may represent a dimeric form of the C-terminal half protein. Western blots of E4.2 (full-length GLUT 1 protein) often also reveal higher molecular weight immunoreactive species. It is widely believed that GLUT 1 may function in dimers or higher order multimers (E.g. Cuppoletti and Jung, 1981, Leib and Stein, 1971, Hebert and Carruthers, 1991). On the expression of the GLUT 4 glucose transporter in insect cells using the baculovirus expression system, Woon et al (1991) found a similar phenomenon occurring on Western blots of their protein. That is, from a monomeric GLUT 4 molecule with an apparent M_r of 38 kDa, a higher molecular weight immunoreactive species was also apparent, at an M_r of 84 kDa. Their conclusion was also that this form was "presumably" a dimeric form of their transporter molecule.

These observations were the basis for the theory upon which the experiments of this chapter were founded. The theory was also based on the work of Bibi and Kaback, 1990, Kaback et al, 1990, and Kaback, 1991 . They studied the *lac* permease protein of *E.coli*. This protein is responsible for the transport of b-galactoside against its concentration gradient, by harnessing the energy from the electrochemical hydrogen ion gradient, in a coupled translocation system. Like GLUT 1, this membrane protein has a putative 12 membrane spanning domains, and is more than 80% helical in structure. At 417 amino acids, its length also is similar to that of GLUT 1. The gene (*lac* Y) was divided into two approximately equal halves, and the two halves (each representing a six transmembrane domain polypeptide) were individually sub-cloned. On the independent expression of the C terminal half, no protein could be detected. On the independent expression of the N-terminal half, protein

could not be consistently detected. Their conclusion was that, as incomplete parts of a whole protein, the individual halves were being proteolysed by the cells. On the simultaneous expression of both polypeptides however, both fragments could be detected by immunoblotting and radiolabelling. In addition to this, no increase in lactose transport was detectable in cells harbouring plasmids containing only one of the halves. Lactose transport, at a rate of around 30% of normal was detected in cells expressing both proteins. Their conclusions from these findings were that an association was occurring in the membranes, between the separate but complementary polypeptides, which prevented or reduced the proteolysis of both, and resulted in a catalytically active complex. However, the observation of any "dimer" or "whole *lac* permease" bands on Western blotting, was not reported.

An equivalent co-expression approach has also since been demonstrated to return function in truncated muscarinic receptors (Maggio et al, 1993). Of a putative seven membrane spanning domain structure, the N-terminal four, including a long connecting loop usually between TM4 and TM5 were expressed in COS-7 cells. The remaining C-terminal "tail" including the final two transmembrane regions were also expressed using these cells. When the two constructs were simultaneously, but under different genetic control, expressed in COS-7 cells, ligand binding, not detected in the single expressions, was recorded. The ligand binding was found to have properties similar to the wildtype receptor.

The theory on which the work of this chapter was based, is as follows. Figure 6.2 describes this theory diagramatically. If, as has been suggested from the Western blotting results, associations are formed between the C-terminal half molecules, to form 12- membrane spanning domain units (as has been suggested to occur between the two different halves of the *lac* permease protein), and perhaps higher order multimeric units; perhaps N-terminal

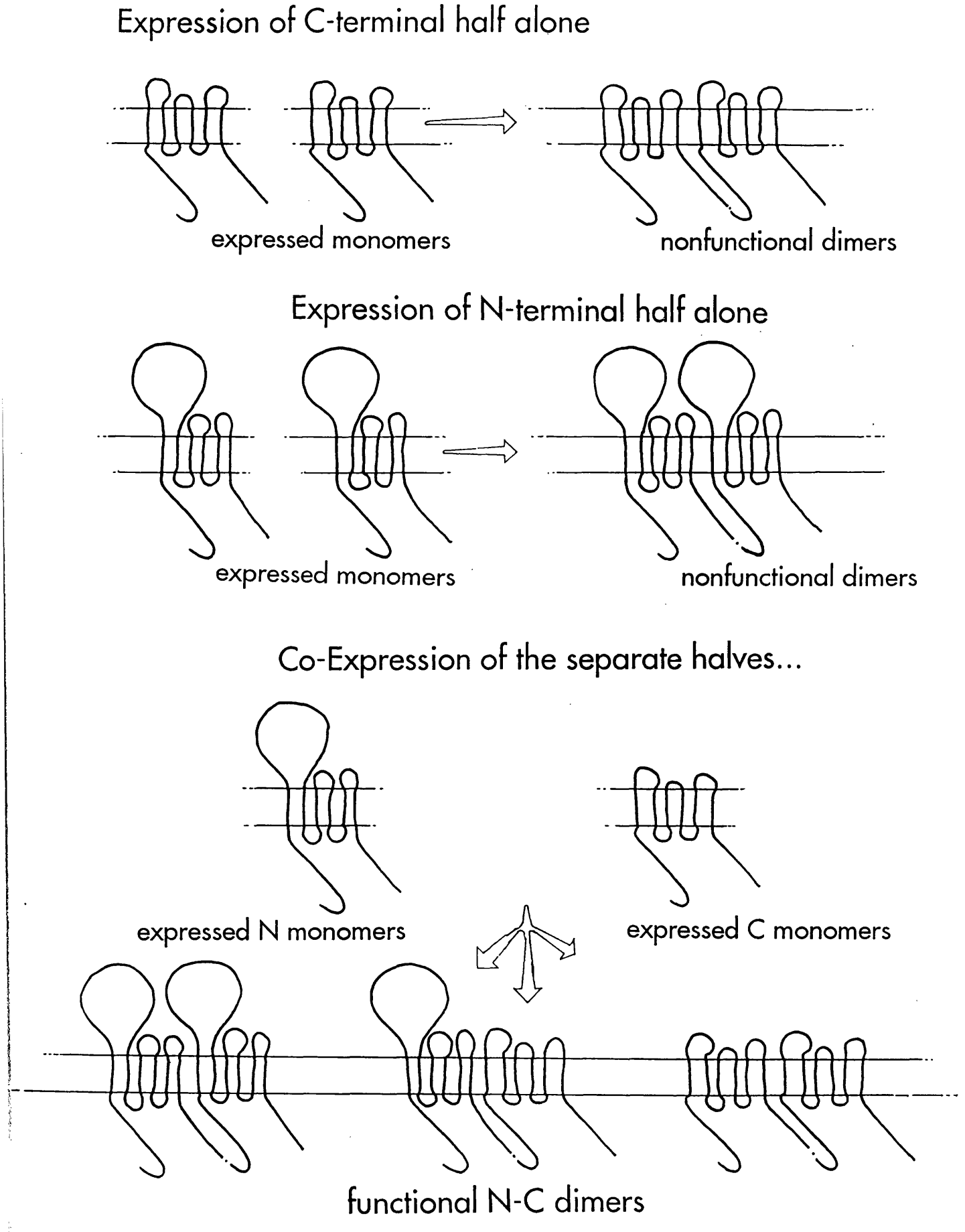

Figure 6.2 Diagramatic representation of the dual infection theory

halves, expressed in the same way, may form similar associations with one another.

If then, in cells infected with the C-terminal encoding virus (R12) C-C associations are formed, and in cells infected with virus encoding the N-terminal half (N1) , N-N associations are formed; perhaps if cells are infected with both R12 and N1 viruses, there may be N-C associations formed. If the two independently expressed proteins are in the correct conformation and orientation, perhaps then, such an association may form a functional glucose transporter, in the same way that lactose transport was detected when the two halves of the *lac* permease protein were individually encoded (Kaback et al, 1990). A functional conformation could be determined, in the same way that function was determined in the GLUT 1 protein expressed whole, using the specific photo ligands (as described in chapter 5).

6.2 THE N-TERMINAL HALF PROTEIN

This theory was approached by the production of a virus encoding the N-terminal half of the protein. The details and results of its independent expression can be seen in chapters three, four and five. The results of the labelling studies were not unexpected. No labelling was detected. There is no body of evidence to suggest that the labelling sites might exist on this part of the transporter molecule.

However, the "dimer" band which was hypothesised as the first part of the theory was detected, in addition to the "monomer" band of the expected M_r. It's detection on Western blots of cells infected with the N-terminal recombinant virus was found to be consistent, and higher molecular weight species were also often detected. Figure 6.3 shows this "dimer" band clearly. At around 53 kDa, it is apparently approximately twice the size of the 27 kDa N-terminal monomer protein. It shows a slightly lesser mobility than the E4.2 protein.

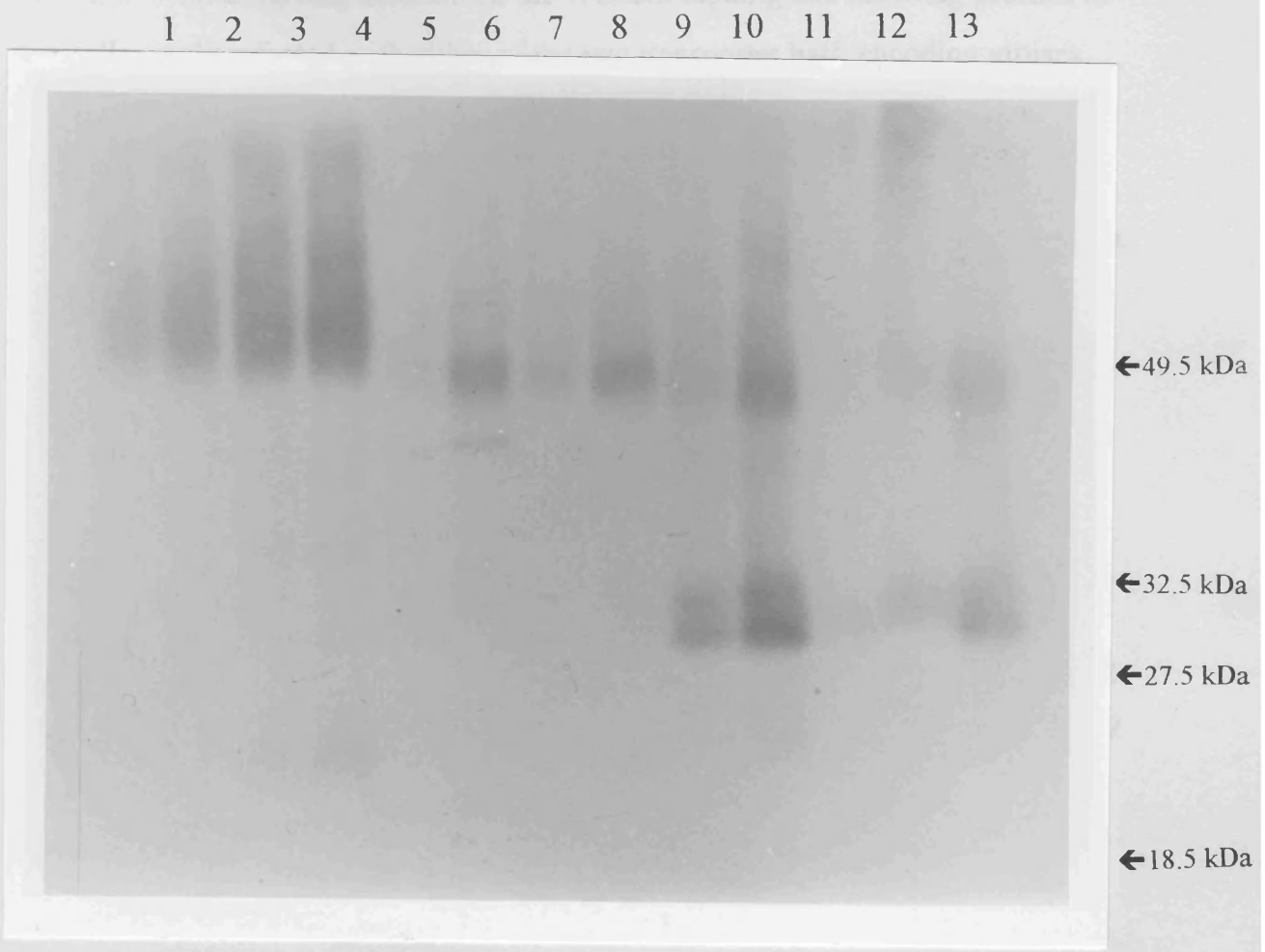

Figure 6.3 Western blot of 12% SDS-PAGE - using the anti N-terminal half peptide antibody. Lanes 1 to 4 show red blood cell membrane GLUT1. Lanes 5 to 8 show 48 hpi membrane preparations of Sf9 cells infeceted with recombinant bacuovirus E4.2. Lanes 9 and 11 show 48 hpi whole cell lysates, and lanes 10 and 12 show membrane preparations of Sf9 cells infected with recombinant baculovirus N1. Note the presence, in these lanes, in addition to the ~27 kDa band of N-terminal half GLUT1 protein, of an immunoreactive species of ~50 kDa.

6.3 SIMULTANEOUS INFECTION OF CELLS WITH VIRUSES ENCODING BOTH GLUT 1- HALF PROTEINS.

After having determined the Western blotting and labelling profiles of cells singly infected with either of the two transporter half encoding viruses, the way was paved to determine these profiles of cells infected with both viruses simultaneously.

Where singly infected, cells were infected with an average of 1.6 p.f.u. per cell (virus titres determined by plaque assay), in order to ensure the simultaneous infection of a large majority of the cells in the culture; doubly infected cells were infected with an average of 1.6 p.f.u. per cell of each virus, in order to infect as many cells as possible with **both** viruses.

6.3.1 WESTERN BLOTTING

On Western blotting of cells infected with both viruses, detected using the anti C-terminal GLUT 1 antibody, the usual immunoreactive profile of R12 was found. That is, the R12 C-terminal monomer and dimer bands appeared, as well as higher molecular weight material. On blotting with the anti-N-terminal half GLUT 1 antibody, the usual profile of N1 was found. Simultaneous detection with both antibodies simply revealed an amalgamation of the two Western blotting profiles of the singly infected cells. A clear size difference exists between the "dimer" bands of the R12 protein and the N1 protein,(R12 dimer having slightly greater, and N1 dimer having slightly lesser mobility than E4.2 protein) but no intermediately migrating species was found. That is, Western blotting failed to reveal a band that was predicted by the theory as representing an N-C dimeric association. Figures 6.4, 6.5 and 6.6 show lysates and membranes of dually infected cells, the GLUT 1 species being detected by the anti- C-terminal GLUT 1 antibody, the anti-N-terminal half GLUT 1 antibody, and both antibodies (respectively).

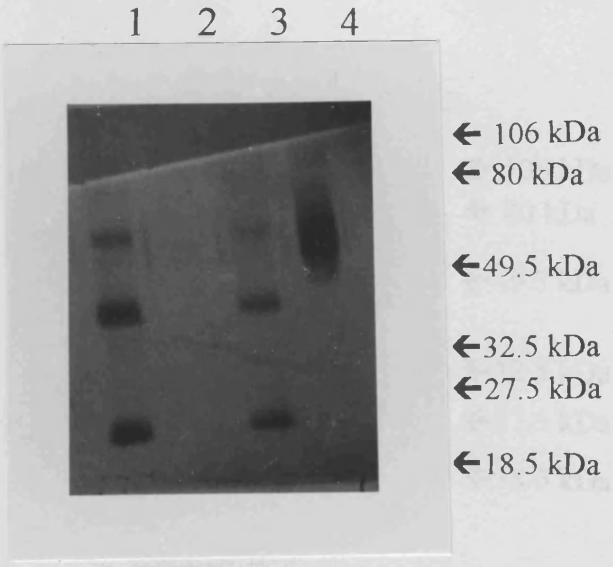

Figure 6.4 Western blot using anti C-terminal peptide GLUT1 antibody. Lane 4 shows red blood cell membranes. Lanes 1, 2 and 3 show Sf9 cell lysates, taken at 48 hpi following infection with; recombinant baculovirus R12 (lane 1), recombinant baculovirus N1 (lane 2); and cells simultaneously infected with both viruses R12 and N1, with a pfu of ~ 1.6 per virus. Equivalent quantities of total protein were loaded in each Sf9 sample lane.

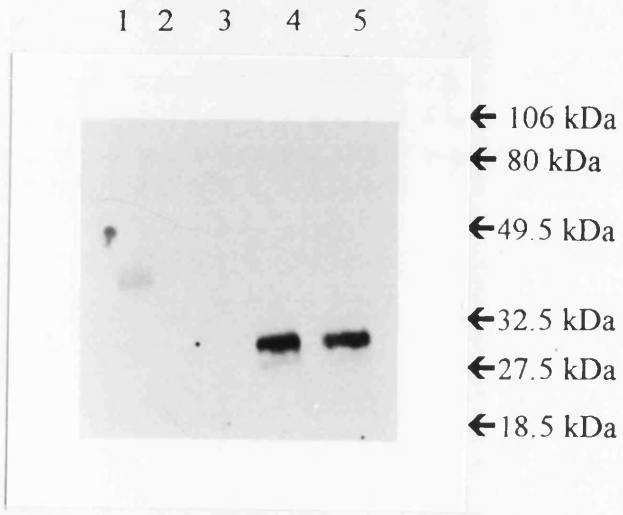

Figure 6.5 Western blot detected using the anti N-terminal half anti-peptide antibody. Sf9 cells were infected with recombinant or "wildtype" baculovirus, at an average of 1.6 pfu/cell. Lysates were made at 48 hpi, and run on a 12% polyacrylamide gel. Lane 1 shows cells infected with AcMNPV-LacZ; lane 2 shows cells infected with E4.2, lane 3 shows cells infected with R12, lane 4 shows cells infected with N1; and lane 5 shows cells infected with R12 and N1 simultaneously.

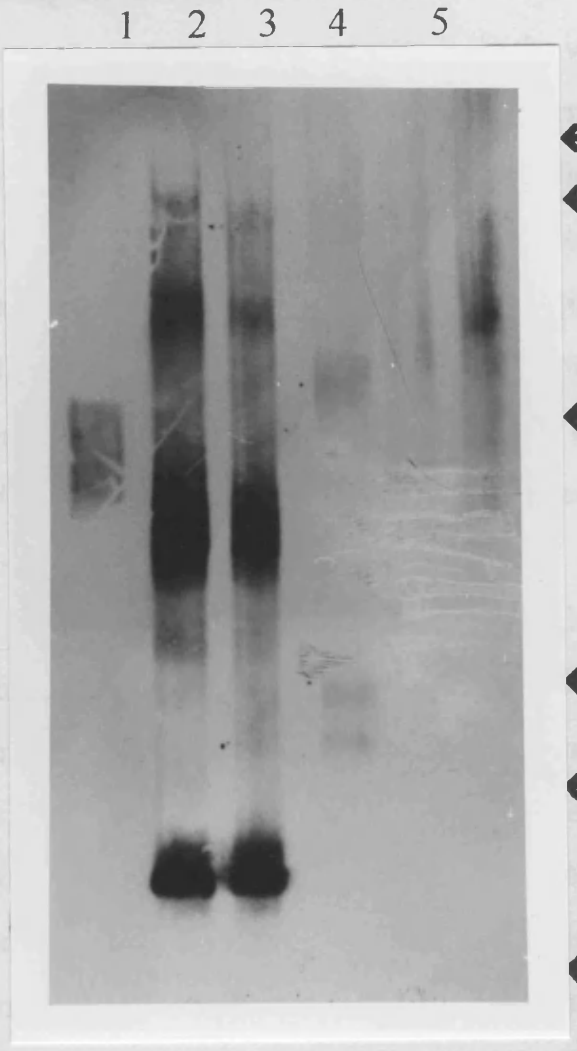

Figure 6.6 Western blot of an SDS-PAG, using anti C-terminal and anti N-terminal half GLUT1 antibodies simultaneously. Lane 1 shows Sf9 cell lysates, taken at 48 hpi, following infection with baculovirus E4.2; Lane 2 shows cells similarly infected with baculovirus R12. Lane 4 shows cells infected with virus N1, and lane 3 show cells dually infected with R12 and N1. Lane 5 shows red blood cell membranes GLUT1.

6.3.2 ATB-BMPA LABELLING STUDIES

Following the theory, N-terminal halves and C-terminal halves should associate by some means, on a one to one basis, in the way which appears to occur in the association of N-terminal halves and N-terminal halves, and C-terminal halves with C-terminal halves. Since no labelling species can be detected in cells expressing only C-terminal half monomers and possible multimers, the hypothesis suggests that although the binding sites are situated on this part of the molecule, perhaps part of the N-terminal half of the molecule is essential for some aspect of the binding. Therefore, the hypothesis would predict that, if formed, the amalgamated N-C dimer species might be labelled with the side - specific ligands.

Cells were infected, in 35mm dishes, as described in chapter 2 , with an avg. infection of 1.6 pfu per cell each of viruses N1 and R12. After 48 hours, a normal labelling experiment was carried out using the photolabel ATB-BMPA. Since the predicted labelling species was expected to be relatively low in abundance, compared to the E4.2 protein, and even the C and N-terminal half molecules, three times the usual quantity of label was used (300μCi), in order to ensure that if a labelling species was present, it would be detected. The experiment was conducted as described in section 2.4.8.

The results are shown in figure 6.7. As is clear from this, a species of protein from the dually infected cells has been labelled with the photoprobe. Labelling at this molecular weight (~25 kDa) is not detected in cells expressing the E4.2 protein, the C-terminal protein alone, the N-terminal protein alone, cells infected with an "irrelevant" β-galactosidase-encoding virus, or cells "mock" infected with TC100 medium only (see chapter 5). However, the hypothesised labelling species was at about 50 kDa (the expected weight of an N-C dimer species). The actual labelled species that was detected has an equivalent M_r of around 25 kDa.. This is more clearly illustrated in figure 6.8,

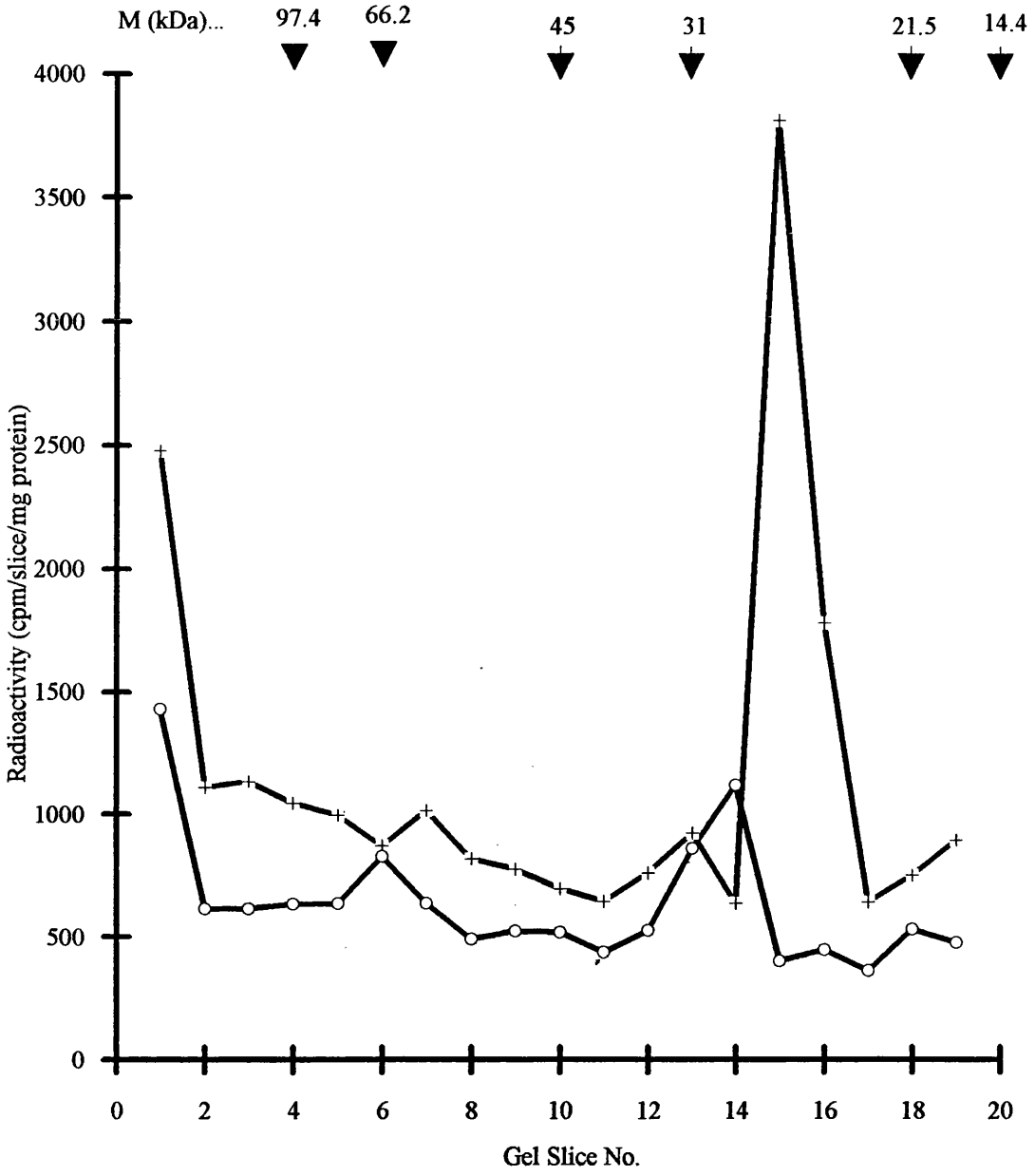

Figure 6.7 ATB-BMPA labelling of Sf9 cells simultaneously infected with recombinant baculoviruses R12 and N1. Cells were taken at 48hpi, and incubated with ATB-BMPA in the presence (o) and absence (+) of 200mM D-glucose. Cells were irradiated for 45 seconds, and immunoprecipitated using the anti GLUT1 C-terminal peptide antibody. Samples were subjected to SDS-PAGE on a 12% acrylamide gel. A labelling peak at ~ 25 kDa is clear.

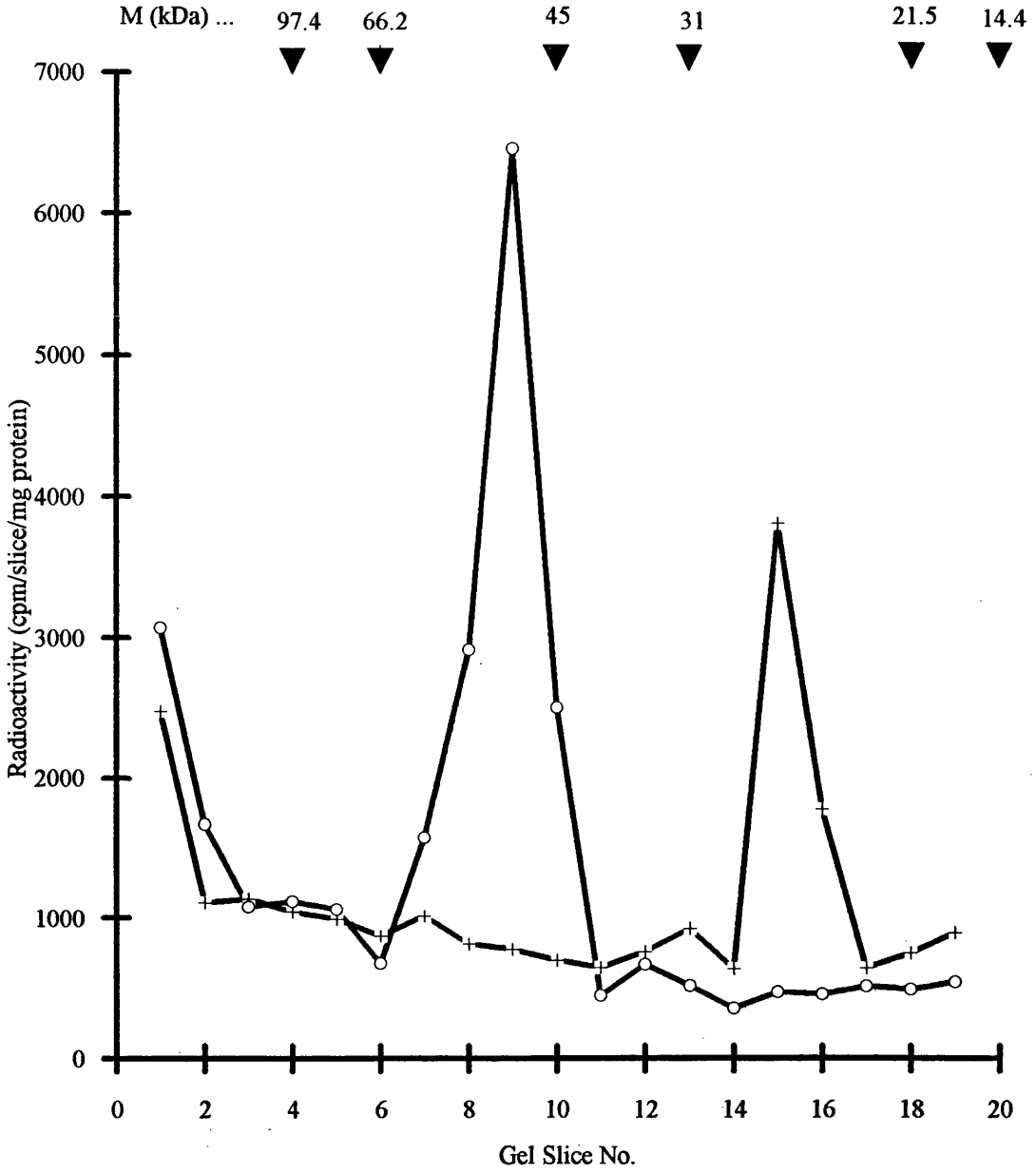

Figure 6.8 ATB-BMPA labelling of dual-infected cells (simultaneous infection with viruses N1 and R12) (+) concomitantly with cells infected with virus E4.2 (o). Labelled cells were immunoprecipitated with the anti GLUT1 C-terminal peptide antibody, and subjected to SDS-PAGE on a 12% acrylamide gel. This illustrates the difference in mobility of the labelling peaks from the two conditions.

which shows the result of an ATB-BMPA labelling of dual infected cells, with a simultaneously performed ATB-BMPA labelling experiment using cells infected with the E4.2 virus. It is clear from its mobility that the species that is labelled by the ATB-BMPA ligand is approximately half the size of the predicted labelling species. The apparent weight of the labelled species corresponds to the molecular weight of the R12 C-terminal monomer species.

This experiment was repeated, and the labelling at this molecular weight (≈ 25 kDa) was found to be reproducible. As is apparent from figure 6.7, less label is found to be associated with the dual-infected cells than cells infected with E4.2. From a series of comparative experiments, it was found that, on average (mean) the dual-infected cells label to about 33% of the cells expressing the full-length protein whole.

Figure 6.9 shows ATB-BMPA labelling of Sf9 cells infected with E4.2, or simultaneously with N1 and R12; where samples were immunoprecipitated with the anti-N-terminal half GLUT 1 antibody. This demonstrates that the anti N-terminal half antibody successfully recognises the E4.2 encoded GLUT 1 protein in an immunoprecipitation. This also demonstrates that the labelled species detected in dually infected cells using the anti-C-terminal GLUT 1 antibody is not immunoprecipitated by the anti-N-terminal half GLUT 1 antibody. That is, the label appears to be associated with the C-terminal half polypeptide, but not with the N-terminal half polypeptide.

6.3.3. CYTOCHALASIN B LABELLING STUDIES

A similar experiment was conducted using the cytochalasin B photolabel (as described in chapter 5 and chapter 2 section 2.2.3.7). Again, the experiment was conducted in the same way as for the three recombinant proteins expressed singly, with the modification of the use of three times the usual quantity of label (48μCi). Figure 6.10 shows the result of such a labelling experiment. There is a clearly defined labelled species at an M_r

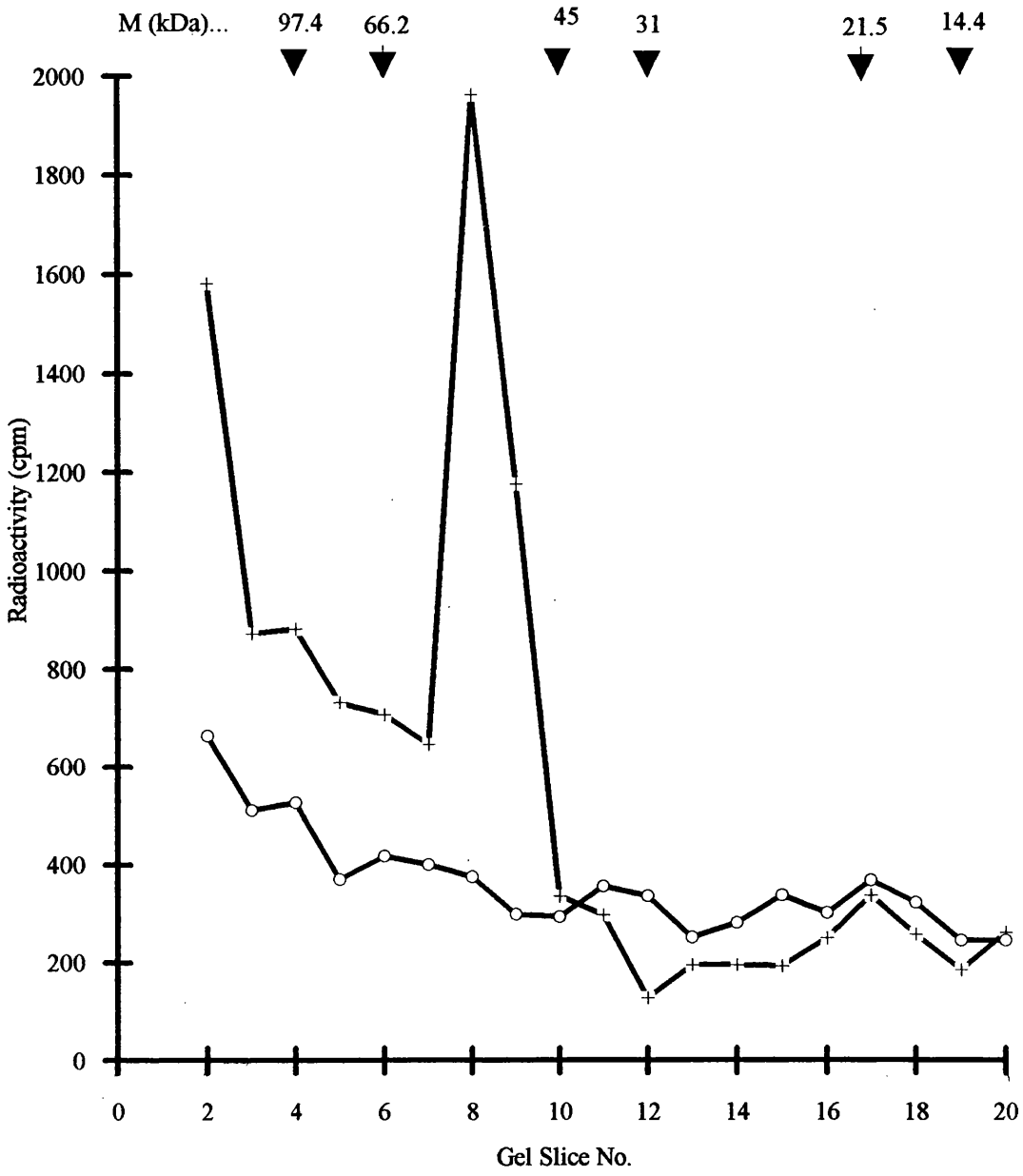

Figure 6.9 ATB-BMPA labelling of dual-infected cells (simultaneous infection with viruses N1 and R12)(o) concomitantly with cells infected with virus E4.2 (+). Labelled cells were immunoprecipitated with the anti N-terminal GLUT1 antibody, and subjected to SDS-PAGE on a 12% acrylamide gel. Although the E4.2 ATB-BMPA labelled species is immunoprecipitated by the anti N-terminal antibody, the dual-infected cells have no labelled species that can be immunoprecipitated using that antibody.

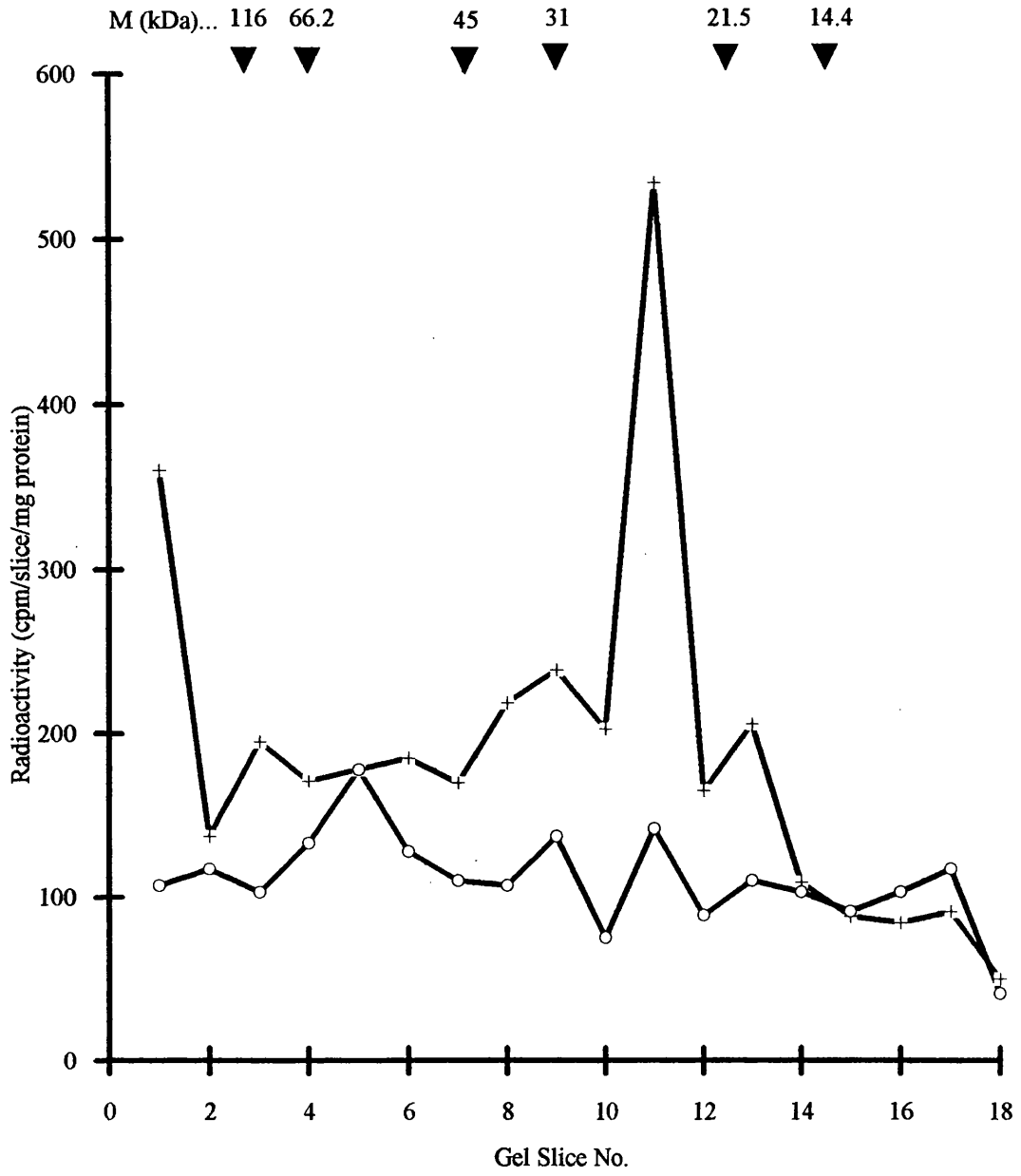

Figure 6.10. Cytochalasin B labelling of cells simultaneously infected with recombinant baculoviruses N1 and R12. Cells, at 48hpi, were incubated with cytochalasin B in the presence (o) and absence (+) of 200 mM glucose, irradiated (as described in 2.2.3.7), and immunoprecipitated using the anti GLUT1 C-terminal peptide antibody. Samples were subjected to SDS-PAGE on a 12% acrylamide gel. A labelled species of ~ 25 kDa can be seen.

corresponding to around 25 kDa. On repeating this experiment, this result was found to be reproducible.

On carrying out a series of comparative labelling experiments between cells expressing the full-length GLUT1 protein (virus E4.2), and cells separately expressing the two halves of the GLUT1 protein (viruses N1 and R12), it was found that the labelling of the GLUT1 protien in the dual-infected cells was about 55% (actual mean 57.7%) that of the whole GLUT1 protein.

6.4 DISCUSSION

The tentative conclusion from these results is that, expressed as a separate protein, the C-terminal half of GLUT 1 can be labelled with ligands specific for both the external and the internal glucose binding sites, if N-terminal protein is present in the cell. Therefore, the general hypothesis outlined in section 6.1 appears to be partially supported. The N-N association predicted in the first part of the theory was apparently detected by Western blotting. The simultaneous expression of independently expressed C-terminal protein and N-terminal protein does result in the binding of ligand by a protein species expressed in the cells as a result of the infection. However, the predicted association of N and C-terminal half proteins could not be detected by Western blotting. However, since the molecular weights of the N-N, C-C, and predicted N-C association species would be expected to differ by only a few kDa, the appearence of the N-C species may be masked in Western blotting by the detection of the N-N or C-C species (depending on which antibody is used in the detection). Also, the size of the protein species predicted to become labelled was the size of the C-terminal monomer, and not the predicted hypothetical N-C dimer species.

Therefore, where Kaback et al (1990) succeeded in producing a functional association of two independently encoded halves of a bacterial transporter protein, without the direct detection of the association *per se*; these results show the successful functional association of the two independently encoded halves of a mammalian transport protein, without the direct detection of an association species.

Since ligands specific to both the internal and the external glucose binding sites have been shown to bind to the C-terminal R12 protein, there seems to be two possible explanations for the conformational state of the C-terminal half protein, or for the N-C association species. Firstly, there may be

two forms of the N-C association, corresponding to transporter protein locked into external- facing form, and some transporters/halves existing in the internal-facing conformation. Alternatively, the association allows the conformational change in the C-terminal half that is necessary for the alternating availability of the two sites for ligand binding. This model would represent a fully functional glucose transport protein.

That the labelling of the C-terminal half in dual-infected cells does not label to the same extent as the full-length GLUT1 protein may be due to a number of points. If the N-C dimer association theory is correct, then, on the basis that that N-C, N-N and C-C associations are present in approximately equal proportions, and based on the observation that monomers of both N-terminal and C-terminal species are still the most highly immunoreactive species in dual-infected cells; the N-C species represents only a fraction of the availabel GLUT1 C-termini in the cells. Even on the basis that there is only a slight apparent reduction in expression of each species in the dual-infected cells as compared to cells infected only with one virus (judged visually from Western blotting), the level of labelling (at 33% and ~55% for the two ligands) is good. On this basis, it would appear that all of the associations give rise to a functional (ie. can be labelled) molecule, which is capable of binding both ligands. However, further investigation would be necessary to achieve this level of analysis.

CHAPTER 7

DISCUSSION

7.1 EXPRESSION OF FUNCTIONAL GLUCOSE TRANSPORTERS IN Sf9 CELLS

The work described in this thesis has demonstrated that by using a recombinant baculovirus, the mammalian GLUT 1 glucose transporter can be functionally expressed in Sf9 insect cells. It has been demonstrated that the insect cells do not innately express any protein species that cross react with the specific anti GLUT 1 antibodies used to detect the baculovirus encoded GLUT 1 proteins. The expression of the full- length protein yields a predominant and broad band on Western blotting following SDS-PAGE at an average M_r of ~50kDa, with smeary high molecular weight immunoreactive products (at ~ 100 kDa, often some higher) being consistently observed at a lower abundance. Optimal expression of the full- length protein product is at around 60 to 72 hpi. However, sufficient quantities of protein for experimentation and analysis were expressed by 48 hpi , and because of significant cell damage observed at 60 to 72 hpi, this time-point was used for further experiments. The expressed protein was demonstrated to be appropriately inserted into membrane, and using side-specific photoreactive ligands, was found to have both internal and external glucose binding sites intact. The expression level of the full length protein was found to be around 150 to 200 pmols GLUT1 protein/mg membrane protein. Although this does not represent a very high level, as is achieved by many proteins expressed using the baculovirus expression system, the amounts present were easily detectable using Western blotting, consistent with levels of other membrane proteins expressed in this system, and fully adequate for any *in situ* analyses.

In 1991, Woon et al successfully expressed GLUT 4 in Sf9 cells in a similar way to the GLUT1 expression described here. The protein was found to be appropriately membrane associated, and the use of a specific antibody to a GLUT 4 extracellular epitope demonstrated that a significant proportion of

the protein was intracellularly located, as is usual for GLUT 4 in its native environments (e.g. in rat adipocytes GLUT 4 is associated with the low density microsomal fraction on subcellular fractionation). Their use of antibodies to an external epitope of the molecule on whole cells also apparently confirmed the appropriate orientation of the molecule within the membrane. Like the GLUT 1 expression described here, Western blotting of Sf9 cells expressing GLUT 4 showed that expression could be detected with specific antibodies by 24 hpi, and reached a maximum expression at around 72 hpi. This was followed by a gradual reduction of expression levels as the viral infection progressed beyond 72 hours. The levels of expression for the GLUT 4 protein, as determined by quantitative immunoblotting were found to be 1.2 nmol/mg membrane protein. This expression level is apparently significantly higher than the levels of GLUT1 expression described in this thesis.

In 1992, Yi et al expressed GLUT 1 in Sf9 cells using the baculovirus expression system. In common with results presented here, and with the expression of GLUT 4 by Woon et al., Yi et al (1992) demonstrated that their GLUT 1 protein was membrane associated. Since there is as yet no reliable subcellular fractionation protocol for insect cells, and in the absence of reliable specific GLUT 1 extracellular epitope antibodies, we were unable to unequivocally determine whether our GLUT 1 protein expressed in insect cells was associated with the plasma membrane, intracellular membranes, or a combination of both. Yi et al, however, using confocal immunofluorescence microscopy, and the production of serial confocal optical sections, were able to demonstrate that GLUT 1 protein was found to be located both at the cell surface, and associated with intracellular membranous structures. A time course of expression showed that the GLUT1 protein expression recorded by that group reached a maximal level at 120 hpi., following a steady increase over the five days followed. This is in contrast to the work described here, where optimal expression was found at around 60 hpi, following a steady increase

from the first appearance of the protein (at 18 to 24 hpi). Following this optimal expression, the levels of detectable GLUT1 protein began to level off, and even to decrease, more closely following the pattern of GLUT 4 expression described by Woon et al (1991). These differences may be due to different multiplicities of infection used by each group (ave. 1.6 pfu/cell for GLUT1 protein described here; ave. 10 for the GLUT 4 of Woon et al, and 5 to 10 for the GLUT 1 of Yi et al.), and the effects of moi and cell growth phase as described in section 1.3.3.

The binding of ligands specific to both the internal, and the external glucose binding sites was demonstrated. However, as is discussed in section 5.1.2, the extent of labelling (with either ligand) did not appear to match the expression levels obtained. That is, although equivalent quantities of RBC GLUT1 and Sf9/baculovirus GLUT1 (determined visually from Western blotting) were used for ligand labelling experiments, the labelling of the insect-cell expressed GLUT1 was apparently only about one third of that of the erythrocyte. Since immunoreactive protein content of the two conditions had been approximately matched, the differences in GLUT1 protein presence cannot account for the differences in labelling that are observed. The differences must therefore be due to either a difference in binding affinity, or reflective of the fact that there may be immunogenic but non-functional transporters being expressed as well as functional ones.

This phenomenon was also observed by Yi et al (1992), where the calculated observed difference was approximately 5.5 fold. The authors suggested that the difference was due to only a proportion of the expressed transporters being capable of ligand binding. A similar phenomenon was observed by Germann et al (1990) on the expression of the human multidrug transporter.

The proposed presence of a mixture of functional, and non-functional GLUT 1 molecules in the insect cell membranes may be the result of different

levels of glycosylation being achieved. That the GLUT1 bands observed on Western blotting are consistently smeary is consistent with the presence of heterologous glycosylation. Heterologous glycosylation is usual for GLUT1, and is observed in the native erythrocyte form, though at a higher average molecular weight than the range observed in the Sf9/baculovirus system. This average molecular weight difference is very likely to be due to a reduced overall level of glycosylation of the insect cell - expressed GLUT1.

As is described in section 1.3.3.1, it is very rare for a glycoprotein expressed using the baculovirus system, to be fully appropriately glycosylated. Insect cells are generally considered to be deficient in the enzymes required for the "decoration" of "trimmed" high-mannose type oligosaccharides. The heterologous glycosylation of many mammalian glycoproteins is a mixture of "trimmed" and "trimmed and decorated" oligosaccharides. Although the presence of more simple oligosaccharides; either high-mannose type, or a mixture of high-mannose and trimmed oligosaccharides has apparently no effect on the biological function of a number of glycoproteins expressed in insect cells (section 1.3.3.1), there are notable exceptions (also outlined in 1.3.3.1). Although there are apparently no cases of completely non-functional glycoproteins being expressed in this way, reduced function, considered to be perhaps due to the reduction in glycosylation, has been recorded: for example, the human multidrug transporter (Germann et al 1990), and the extracellular domain of human nerve growth factor (Vissavajjhala and Ross ,1990).

The expression of GLUT1 by Yi et al (1992), showed that the protein had an apparent molecular weight of around 45 kDa. In contrast with the GLUT1 band described here, a smeary band of around 50 kDa, their band was fairly sharp. Endoglycosidase F treatment yielded a protein of slightly increased electrophoretic mobility, which was barely different from the deglycosylated erythrocyte GLUT1 (at ~46 kDa). This demonstrated that their GLUT1 protein was glycosylated only to a limited extent of the native

erythrocyte form. The relative absence of smearing of the GLUT1 band suggested that their GLUT1 protein was relatively homologously glycosylated. That the GLUT1 protein expression described here is of higher apparent molecular weight, and an immunoreactive band apparently more smeary, than that described by Yi et al, may be indicative of an expression in which a heterologous range of glycosylation (perhaps high-mannose type, and trimmed oligosaccharides) is achieved. However, the glycosylation state of GLUT1 expression described here, was not experimentally determined. Treatment with endoglycosidases, and analysis by Western blotting would be necessary in order to reach any conclusions about the actual state of glycosylation of the expressed protein. The differences in molecular weight and range observed between the GLUT1 protein expressed in the same way but by two different groups may only be technical. That is, a different range of molecular weight standard markers was used by each group, and slight variations in technique of sample and gel preparation are likely to be the case. These technical differences may be sufficient to account for the apparent molecular differences. In order to unequivocally conclude that there are differences in the levels of glycosylation and in the molecular weight of the GLUT1 protein between the different groups (both using the AcMNPV/Sf9 baculovirus system), they would have to be compared directly. In the absence of such a study, of the GLUT 1 protein expressed here, it can be said that some level of glycosylation is apparent (treatment with endoglycosidases in concert shows that the core polypeptide of erythrocyte GLUT1 has an M_r of ~ 38,000 kDa, Haspel et al, 1985), and that that glycosylation is not to the extent of the native erythrocyte GLUT1. The expression of GLUT 4 by Woon et al (1991) showed that this molecule too, had a reduced glycosylation status when expressed in insect cells.

Studies of the importance of glycosylation of the glucose transporter proteins have suggested that the alteration of the asparagine- linked glycosylation can effect the rate of transcription of transporter DNA, but does

not affect glucose uptake (Haspel et al, 1988, on studying GLUT 1 expressed in N-acetylglucosaminyltransferase 1- deficient CHO cells. (Asano et al, 1992,1993). These studies suggested that alterations in the glycosylation of the transporter may affect the formation of a structure with a high affinity for glucose, or the targeting of the protein to the membrane compartments appropriate to its isoform. The apparent observation (here, and by Yi et al, 1992) that only a proportion of the expressed GLUT1 molecules are capable of ligand binding at the glucose binding sites, (or that the binding is of a reduced affinity compared to native GLUT 1), is consistent with these suggestions.

The evidence for the effects of altered, particularly reduced, glycosylation on the biological function of a glycoprotein is largely based on the evidence of proteins being expressed in systems deficient of one or more of the enzymes required for full glycosylation. It is therefore largely circumstantial and rather indirect. Much more research is required in this important area, before definitive statements of cause and effect can be attached to the glycosylation status and functional status of glycoproteins.

7.2 GLUCOSE TRANSPORT IN BACULOVIRUS-INFECTED Sf9 CELLS.

The functional status of the GLUT1 glucose transporter expressed in Sf9 cells was determined by the use of two transporter side-specific ligands. These results are described in chapter 5.

Glucose transport measurements, thought exhaustively tried, were found to be unsuccessful, and therefore not an appropriate method of determining glucose transporter function in this system. The conclusions reached by Yi et al (1992) on their expression of GLUT1 using the Baculovirus expression system were in agreement with this. That is, that by the time in an infection that a measurable amount of transporter protein is expressed by the cells, the cell membranes are beginning to suffer as a result of the viral infection, and become "leaky".

This phenomenon is not restricted to glucose transport proteins. For example, Germann et al (1990) expressed the human multidrug transporter, which usually functions to confer multidrug resistance to a cell. It was not possible to demonstrate this when the transporter was expressed in insect cells using the baculovirus system. They also concluded that this was due to the cells becoming leaky during the course of the infection. The functional status of this molecule was determined by ligand binding, and found to be functionally similar to its authentic counterpart.

Although the binding of ligands to each glucose binding site of the baculovirus/insect cell GLUT1 molecule is good evidence for the functional status of the transporter, empirical evidence of actual transport would be necessary for fully definitive evidence that the molecule functions as a glucose transporter. This could be achieved by reconstitution experiments in which the Sf9/baculovirus expressed transporter is put into intact membrane systems, where the integrity of the original host membrane is unimportant. GLUT 1

from erythrocytes have been reconstituted into non-native membrane environments (for example; Kasahara and Hinkle, 1977; Hinkle et al, 1979; Baldwin et al, 1980; Baldwin et al, 1981; Chin et al, 1986). Baldwin et al (1981) described the reconstitution of GLUT 1 into vesicles such that each vesicle contained an average of one GLUT1 molecule. Although they found that the transport activity of the reconstituted erythrocyte GLUT1 was only ~5% of that of intact erythrocytes under similar conditions, the activity was readily measurable, and was found to be due to a general reduction in activity of all molecules, rather than the loss of function of a proportion of them. In this case, the detection of transport of reconstituted insect cell-expressed transporters would conceivably be possible.

In view of the evidence that has been obtained, and that a vast majority of foreign proteins, even glycoproteins, expressed in insect cells using the baculovirus system are found to be biologically functional, often to the same level of the protein in a native environment, it is quite likely that transport activity, in a reconstitution system , would be detected, and that that activity would match the ligand binding that has been measured. If measurements of hexose transport could not be made in such a system, this would suggest that perhaps the apparent reduced affinity or presence of non-functional molecules as compared to the transporter in the erythrocyte environment, may actually indicate the expression of two forms of transporter. That is, one form that is locked into an inward-facing conformation, and one form that is locked into an extracellular-facing conformation; with no conformational change possible between the two. Given the circumstantial evidence that most other proteins expressed using the baculovirus expression system are found to be biologically functional, and that a degree of function has been demonstrated for the GLUT1 protein, by way of the ligand binding; it is perhaps more likely that hexose transport would be measurable in a reconstituted membrane system.

7.3 FUNCTIONAL ARRANGEMENT OF GLUCOSE TRANSPORTER MOLECULES.

7.3.1. ASSOCIATION OF TRANSPORTER MOLECULES

At the onset of this project, it was intended that the C-terminal half of the GLUT1 protein would be expressed in Sf9 cells independently. In the light of all of the evidence pointing to the C-terminal half as the "functional end" of the transporter, is was hypothesised that it might function as a transporter when expressed alone. Chronologically fairly early, it was clearly demonstrated that the C-terminal half expressed alone was not able to bind ligands at either the internal, or the external glucose binding sites. Chapter six describes the theory behind the production of an N-terminal half-encoding baculovirus, and its co-expression with the C-terminal half to produce a functional unit. The reasons why it was considered that this might work, and the description of a similar experiment conducted in *E.coli* bacterial cells (Bibi et al, 1990; Kaback et al, 1990) are also fully discussed in chapter six.

One of the bases for the development of the dual expression hypothesis was the observation of higher molecular weight material on Western blots of the independently expressed C-terminal half protein. Higher molecular weight species were also found on many Western blots of the full-length transporter. It is quite evident from the literature that the appearance of apparent multimeric forms of a number of proteins has been reported. The observation of higher molecular weight species on Western blotting of the expression of GLUT 4 in baculovirus was also made (Woon et al, 1991).

A good example of the detection of a multimeric form of a protein on SDS-PAGE is that of the Sf9/baculovirus expression system expressed serotonin receptor, of Ng et al (1993). On Western blotting, the monomeric form, at ~42 kDa was the most prominent. The proposed dimer band at ~95

kDa was less prominent. Both bands were found to be immunoprecipitated by a specific antibody for the serotonin receptor. However, where, on the expression of glucose transporters using the baculovirus system no functional status could be attributed to the higher molecular weight species, Ng et al (1993) found that photolabelling with a serotonin receptor specific ligand labelled species at **both** molecular weights. Although apparent dimers have been described for a number of proteins, this example positively confirms (beyond immunological reactivity) the identity of the higher molecular weight species as actually being a multimeric form of the protein in question.

As is described in chapter one (section 1.1.5 in particular) it has been suggested that the functional unit of the GLUT1 molecule is as a multimer; either a dimer, or a tetramer. There is much evidence to support this view. If there is a close interaction between GLUT1 molecules, then it may seem feasible that the highly hydrophobic nature of the overall GLUT1 protein, along with whatever forces are involved in the maintenance of multimeric units, may cause aggregation on SDS-PAGE such that the multimers would appear on the gels. Hebert and Carruthers (1992) have suggested that the maintenance of the proposed homotetrameric structure may be stabilised by intramolecular disulphide bonds, which are preserved particularly in the absence of a reductant by the hydrophobic nature of the GLUT1 protein (samples for SDS-PAGE described in this thesis were solubilised in a 6M urea sample buffer without β-mercaptoethanol, and were not boiled before loading onto the gels). They propose that there are up to two intramolecular disulphide bonds per monomer pair, and that the establishment of the native structure is established prior to translocation to the plasma membrane. They propose that the intra-subunit disulphide bonding promotes the co-operative subunit interactions that stabilise transporter structure and function.

Associations between different molecules have been reported from dual expression studies of proteins using the baculovirus system. The Simian Virus 40 large T-antigen was dually expressed with mouse p53 protein in Sf21 cells (O'Reilly and Miller (1988). During infection with Simian Virus 40; the large T-antigen normally associates with the host cell p53 protein. Such an association is also recorded when both are simultaneously expressed in insect cells. Another example is the expression, from two different recombinant baculoviruses, of two Influenza viral polymerase proteins results in the formation of a functionally intact association, as would occur in a host cell when both proteins are encoded by the Influenza virus (St. Angelo et al, 1987). Therefore, the insect cells apparently permit interactions between proteins as would occur in their authentic environment.

If intra-molecular disulphide bonding between GLUT1 molecules so readily occurs, and that there is more than one such bond per molecule, as suggested by Hebert and Carruthers (1992), then the appearance of dimers of the C-terminal half is not unreasonable. If an appropriate conformation of the C-terminal monomers is achieved within the membrane, then it may be reasonable to assume that the mechanisms of molecular recognition responsible for the formation of intra-subunit disulphide bonds within the intact molecule should also apply to the association of C-terminal monomers. If the suggestion that there are at least two such bonds responsible for the association of intact GLUT1 molecules is correct, then it is possible that at least one associates the C-terminal half of one molecule to the C-terminal half of another; and that another associates the respective N-terminal halves. Since we have demonstrated the existence, on Western blotting of cells infected with virus encoding only the N-terminal half of GLUT1, of apparent N-terminal dimers, then some kind of intra-molecular association is clearly taking place.

Pessino et al (1991), as discussed in chapter one, have presented evidence that GLUT1 functions in oligomeric formations. They describe the

expression of GLUT 1:GLUT 4 chimeric molecules, in cells endogenously expressing GLUT1, and the successful immunoprecipitation of native GLUT1 molecules using specific GLUT4 antibodies. By the nature of the construction of the chimeric molecules, they concluded that a major determinant of oligomerisation of GLUT 1 is located within the first 199 residues. The work described in this thesis apparently contradicts this, if the higher molecular weight species of the half molecules expressed separately are to be believed to be dimers. However, although Pessino et al (1991) suggest a major determinant to be located within the first 199 residues, they do not suggest that there is not also a determinant located within the remainder of the molecule.

The work of Popot and Engleman (1990) on protein folding and oligomerisation uses the bacteriorhodopsin molecule as a model. They discuss the nature of forces and bonds responsible for holding membrane proteins in the correct conformation. The bacteriorhodopsin molecule can be cleaved into two parts (a five transmembrane portion, and a two transmembrane-domain spanning portion) by a single cleavage. The two fragments can be completely denatured, and re-folded in separate vesicles. Upon fusion of the two types of vesicle, the two fragments successfully re-form an appropriate conformation for the binding of retinal. That is, the native structure is apparently completely re-formed, and a covalent link between the two fragments is not required for the molecule to refold properly.

The findings of Popot and Engleman (1990) might suggest that the association of the independently expressed portions of the glucose transporter may be due to their adoption of conformations that are sufficiently similar to their structures in the entire GLUT 1 protein, that recognition and correct association occur purely on this basis.

The successful, albeit non-functional, independent expression of the two halves of the GLUT1 glucose transporter serves to support part of a theory constructed by Mueckler and Lodish (1986). They proposed that the GLUT1

glucose transporter, having no cleavable signal sequences, has at least two signal sequences responsible for the direction to, and insertion into the membrane. They propose that such a signal exists at least once in the N-terminal half, and at least once in the C-terminal half of the molecule. They propose that these signals target the polypeptide to the membrane, and initiate insertion into the membrane co-translationally; each sequence initiating the insertion of the polypeptide area immediately adjacent to it, as soon as it itself has been translated. That both the N-terminal polypeptide and the C-terminal polypeptide expressed in the insect cells are both found to be membrane associated, and that together they associate to form a functional unit, confirms that there is at least one independently functional membrane-insertion signal sequence in each half of the molecule.

Molecular modelling and mapping studies suggest that GLUT1 may exist as a bilobular structure, consisting of two closely packed bundles of six helices, corresponding to the N-terminal and the C-terminal halves of the molecule (Hodgson et al, 1992). The flexibility of the long central cytoplasmic loop between TM 6 and TM 7 allows the packing in these two distinct domains, and the relatively short linker regions between the other transmembrane segments suggest a close packing of helical bundles in each of the two halves. The dual expression studies described in this thesis support this view. It is demonstrated that although it is the C-terminal half of the molecule to which the ligands bind, the presence of the N-terminal half of the molecule is required in order for the binding to take place.

It is suggested that the N-terminal half, or part of it, provides a packing surface for the C-terminal half, such that this part of the molecule can adopt an appropriate conformation for the binding of ligands. From studies of the role of glycosylation of the glucose transporter molecule already discussed, it has been strongly suggested that glycosylation, which is found only associated with Asn 45 of the N-terminal half, may be important in the maintenance of a functional

conformation. Based on this, the glycosylation of the N-terminal half may perform a key function in the formation of a ligand binding conformation of the C-terminal half.

In order to more closely define the relationship between the C- and N-terminal halves of the molecule, a series of constructs could be made which consist of selected areas of the N-terminal half only. Viruses from these constructs could then be co-expressed in insect cells with the C-terminal half virus R12. A full six-membrane spanning domain N-terminal construct with a single amino acid substitution at Asn 45 to produce a non-glycosylated form may be one of the simplest, to test the hypothesis that the glycosylation of the molecule is important in the formation of a functional conformation. The production of a series of constructs having sequential deletions of the membrane spanning helices may also be useful. However, not all protein products may be viable, or may not be correctly targeted, due to the absence of signal sequences etc. The analysis of membrane association of such constructs may be helpful in determining the localisation of the signal sequences involved in the membrane insertion of the N-terminal half. The close analysis of whether "dimer" bands are present on Western blotting of cells singly infected with the sequentially truncated N-terminal constructs may also provide indications about the localisation of intra-molecular interactions within this half of GLUT 1.

Figure 7.1 shows a cartoon of the three-dimensional molecular modelling of the GLUT 1 molecule based on the two dimensional model, and considerations such as the lengths of the putative helix-connecting loops (Hodgson et al, 1992; Gould and Holman, 1993). This structure (also discussed in section 1.1.3.1) is a bilobular arrangement of two six-transmembrane-helix bundles, corresponding to the N- and C-terminal halves. This model was constructed before the commencement of the work described in this thesis.

Figure 7.1 Cartoon representation of the proposed three-dimensional structural arrangement of GLUT1, showing the proposed bilobular structure of the two six-transmembrane domain spanning halves. The N-terminal half is shown in orange, and the C-terminal half in red.

The large central loop, and the very short nature of the remaining α-helix connecting loops may be indicative of a bilobular tertiary structure. The length and sequence of many of the connecting loops, especially at the cytoplasmic surface, are highly conserved, at seven to 15 residues, and are generally shorter at the cytoplasmic surface. This suggests a very close packing of the six helixes of each domain, rather than a single bundle of twelve helixes. The slightly shorter loops on the cytoplasmic surface suggest a closer association of the helixes at this side of the molecule. A structure of this nature has been observed in the twelve -membrane- spanning domain protein of *E.coli*, lactose permease, by low resolution electron microscopy (Li and Tooth, 1987). A molecular model of the two domain packing of GLUT1 is illustrated in figure1.2

7.4 THE FUTURE OF GLUT 1 AND THE BACULOVIRUS EXPRESSION SYSTEM.

The further elucidation of the packing arrangement of GLUT 1 with respect to its two halves has been discussed in section 7.3, and is likely to be the primary route to be followed with respect to the expression of GLUT 1 in insect cells using the baculovirus expression system.

Although the expression levels of the three GLUT 1 protein products were not as high as have been reported for many proteins expressed using the baculovirus system, the levels achieved were not low in comparison with other membrane associated glycoproteins. On consideration of this, and the relatively low virus titres achieved (in comparison to other reported recombinant baculoviruses), the expression levels achieved are good. With the use of a simple purification technique for the transporter, it may be possible to produce sufficient quantities of GLUT 1 expressed in baculovirus to allow further examination of the protein structure, such as crystallisation trials with a view to X-ray crystallography.

The insertion of a short section of DNA encoding five or six histidine residues, into the central loop region of GLUT 1 would not be expected to have an effect on either the structure or function of the molecule. The expression of a protein with a polyhistidine section allows a one-step purification method to be carried out. A metal ion affinity column, where (for example) nickel ions are immobilised on a solid support allows the purification of proteins bearing a polyhistidine motif. If the protein could be satisfactorily eluted from the column and purified to a non-denatured state, this could prove to be a suitable substrate for structural analysis. This work is currently in progress in Dr Holman's laboratory.

Since the C-terminal half of GLUT 1 expressed alone has been demonstrated to be correctly inserted into the membrane, and, in the presence

of the N-terminal half, able to take up an appropriate conformation for the binding of ligands, this too may be a suitable substrate for three dimensional structure determination. The use of a specially created baculovirus transfer vector such as "pBlueBacHis" (Invitrogen, R & D Systems Europe Ltd., Abingdon, OX14 3YS) allows, by subcloning of the desired gene or fragment, the addition of a polyhistidine tail to the N-terminus of the expressed protein. This could be purified using a metal ion column, as described. Although there is some question about the conformational state of the structure of the C-terminal half in isolation, the purification and renaturation of this molecule may be more successful than the larger full-length GLUT 1 molecule.

For whatever particular purpose the baculovirus system expressed GLUT 1 protein and fragments may be used, this project has demonstrated the potential for this system for the expression of glucose transport protein. The demonstration that the C-terminal half, known to bear the ligand binding domains of the transporter, requires the N-terminal half in order to adopt the correct conformation for ligand binding, may only be the first step in a series of important findings about glucose transporter structure and function.
The construction of new GLUT 1 fragment-encoding baculoviruses may provide much more information about the structure-function relationship of the GLUT 1 glucose transporter; and the purification of large quantities of transporter protein may be possible, to enable three-dimensional structure determination of the molecule.

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APPENDIX I PST I DIGESTED LAMBDA DNA SIZING